G PROTEIN COUPLED RECEPTOR SIGNALLING

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Dedicate to my parents with love and gratitude.

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ABSTRACT

The present studies were undertaken to examine the biochemical mechanisms of transmembrane signalling of the cloned 5-HT1A, dopamine-D1, D2S and D2L receptors in GH4C1 pituitary and Ltk- fibroblast cells. It was found that when expressed in GH4C1 cells, all receptors examined exhibited similar signalling phenotype consistant with what is found in neurons where these receptors are normally expressed. When expressed in Ltk- cells, however, all receptors, in addition to inhibition or stimulation of adenylyl cyclase, were shown to induce a novel activation of phospholipase C activity. Acute activation of protein kinase A or C differentially and selectively modulate distinct signal transduction pathways mediated by these receptors. Furthermore, it was observed that αo specifically triggers closure of calcium channels while αi2 specifically initiates inhibition of cAMP synthesis; there are clear differences in coupling to Go and Gi2 between dopamine-D2S and D2L receptors. In addition, it was observed that different receptors may link to distinct αi subunits to regulate different levels of adenylyl cyclase activity in GH4C1 cells, and the coupling of receptor to αi subunits may change when a Gs coupled receptor is activated.

RÉSUMÉ

Cette thèse examine les mécanismes de transuction membranaires associés aux récepteurs de type 5-HT_{1A} et dopamine de type D_1 D_{2S} et D_{2L} dans des cellules transfectées de type GH4C1 dérivées de l'hypophyse et de type fibrobastiques Ltk. Dans les cellules GH4C1, les systèmes de transduction associés à chacune des classes de récepteurs transfectés est en accord avec les résultats obtenus dans les tissus neuronaux exprimant naturellement ces récepteurs. Toutefois, dans les cellules Ltk, en plus de la modulation habituelle de l'activité de l'adélynate cyclase, tous les récepteurs transfectés stimulent l'activité de la phospholipase C. De plus, l'activation des protéines kinases A ou C affecte différentiellement et de façon sélective, des systèmes distincts de transduction associés à ces diverses classes de récepteurs. Nous avons aussi observé que la protéine G de type αo induit spécifiquement la fermeture de canaux calciques alors que celle de type $\alpha i2$ amorce l'inhibition de la synthèse d'AMP cyclique. D'importantes différences de couplage des protéines Go et Gi2 ont aussi été observées quant aux récepteurs D_{2S} et D_{2L}. Finalement, nous avons montré que diverses classes de récepteurs peuvent s'associer à des sous-unités α distinctes permettant ainsi de contrôler des niveaux différents d'activité de l'adénylate cyclase dans les cellules GH4C1. De plus, le couplage d'un récepteur aux sous-unités α peut changer lors de son activation.

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STATEMENT OF CONTRIBUTIONS

The thesis is comprised of six different papers. The experimental work described in the papers was designed and performed by Y.F.L., with the following exceptions:

1. In Chapter IV, the GH4-hD1 and L-hD1 cell lines were made in Dr. Civelli's laboratory and ligand binding assay was performed in Dr. Civelli's laboratory.

2. In Chapter V, the RGB-2 and L-hD2L cell lines were made in Dr. Civelli's laboratory and ligand binding assay was performed in Dr. Civelli's laboratory.

3. In Chapter VI, western blot was performed in Dr. Rasenick's laboratory.

[a]reprinted from the Guideline Concerning Thesis Preparation, Faulty of Graduate Studies and Research, McGill University, January 1993

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ABBREVIATIONS

G proteins,	guanine nucleotide-binding regulatory proteins
Gs,	stimulatory G protein
Gi	inhibitory G protein
Go	other G protein identified from brain
Gt	transducin
Golf	G proteins identified in olfactory system
Gq	G protein link to activation of phospholipase C
GTPγS	guanosine 5'-3'-O-(thio)triphosphate
PLC	polyphosphoinositide-specific phospholipase C
PI	phosphatidylinositol
IP3	inositol, (1,4,5,)-triphosphate
PIP2	phosphatidylinositol (4,5)-bisphosphate
DAG	diacylglycerol
cAMP	3,'5'-cyclic adenosine monophosphate
PKC	protein kinase C
PKA	protein kinase A
PTX	pertussis toxin
CTX	cholera toxin
5-HT	5-hydroxytryptamine, serotonin
8-OH-DPAT	8-hydroxy-(2-N-dipropylamine)-tetralin
VIP	vasoactive intestinal peptide

TRH	thyrotropin-release hormone
D1	dopamine-D1 receptor
D2S	the short form of dopamine D2 receptor
D2L	the long form of dopamine D2 receptor
fMLP	formyl-Met-Leu-Phe
TSH	thyrotropin
LH	luteotropin
N-terminus	amino-terminus
C-terminus	carboxyl-terminus.
RT-PCR	Reverse transcription-polymerase chain reaction
[Ca++]i	Free intracellular calcium concentration
5-CT	5-carboxamidotrytamine
ß-ARK	ß-adrenergic receptor kinase
α	G protein alpha subunit
ß	G protein beta subunit
γ	G protein gamma subunit

CHAPTER I RESEARCH OBJECTIVES

The G protein coupled receptor transmembrane signalling system includes at least three membrane-bound components: a cell surface receptor, guanine nucleotide-binding proteins, G proteins, and one or more effectors (Gilman, 1987; Stryer & Bourne, 1989; Ross, 1991; Birnbaumer et al, 1990). Agonist-bound receptor activates G proteins by enhancing the rate of exchange of GTP to GDP bound to the α subunit, resulting in the dissociation of α-GTP and the ßγ dimer (Gilman, 1987; Bourne et al 1991). The activated α-GTP complex in turn alters the activity of effectors, as in hormonal stimulation or inhibition of adenylyl cyclase, by αs and αi respectively, as in hormonal closure of calcium channels by αo or opening of potassium channels by αi3, and as in hormonal stimulation of phospholipase C and A2 activities by αq, etc., (Gilman, 1987; Birnbaumer et al, 1991; Bourne et al, 1990).

Subsequently, the agonist-activated receptor undergoes a rapid loss of receptor responsiveness despite the continuing presence of the agonist, a process called receptor desensitization (Lefkowitz et al, 1990; Dohlman et al, 1991; Kobilka, 1992). Several biochemical changes are involved in the rapid desensitization of receptors, including receptor sequestration and phosphorylation of signalling components, eg. receptor, effectors, and/or G proteins. In many cases, phosphorylation appears to uncouple the receptor and G protein providing a negative feedback signal to limit the magnitude and duration of receptor signalling. The kinases involved in receptor phosphorylation belong to the serine/threonine kinase family, and include protein kinases A and C, and receptor-specific protein kinases, eg. ß-adrenergic receptor kinase (ßARK). The role of phosphorylation in desensitization of ß-adrenergic and rhodopsin receptors has been extensively studied (Benovic, et al 1990; Lefkowitz et al, 1990;

Dohlman et al, 1991). However, the role of protein kinases in the desensitization of Gi/Go- and Gq-coupled receptors remains to be further elucidated.

The objective of the present studies was to further elucidate the biochemical mechanisms of transmembrane signalling of PTX-sensitive G protein coupled receptors: 1) the cell-specific signalling of hormone receptors; 2) the regulatory roles of protein kinase A and C in these receptor-mediated multiple signal transduction pathways; 3) the roles of distinct G proteins in the specific receptor/effector interactions.

Both 5-HT1A and dopamine D2 receptors are inhibitory receptors: they initiate inhibition of adenylyl cyclase, opening of potassium channels and closure of voltage-dependent calcium channels via pertussis toxin (PTX) sensitive G proteins, Gi/Go (Frazer et al, 1990; Civelli et al, 1991 & 1993; Sibley & Monsma, 1991). By contrast, the dopamine-D1 receptor is a stimulatory receptor which induces stimulation of adenylyl cyclase (Civelli et al, 1991 & 1993). In order to investigate G protein-coupled receptor transmembrane signalling mechanisms in detail, the cDNAs of these receptors were individually transfected into two different cell systems, the rat GH4C1 pituitary lactotroph cells and mouse Ltk- fibroblast cells. Both of these cell types lack detectable endogenous 5-HT1A, dopamine-D1 and D2 receptors and thus provide an appropriate background to analyze the signalling of each receptor subtype. GH4C1 cells are electrically excitable cells which have many neuronal properties, including expression of variety of the voltage-dependent calcium and potassium channels, synthesis and secretion of growth hormone and prolactin , which are regulated by several endogenous G protein coupled receptors, including somatostatin, muscarinic-M4, VIP, and TRH receptors (Dorflinger & Schonbrunn, 1983; Yatani et al, 1987; Enyeart et al, 1990); they also express G protein αo and $\alpha i1$ subunits

which were only found in neurons (Largent et al, 1988). In contrast, Ltk- fibroblast cells have a more rapid growth rate than GH4C1 cells; and they do not express any of voltage-dependent calcium channels or potassium channels (Liao et al, 1990), and G protein $\alpha i1$ and αo subunits are also absent in Ltk- cells (Y.F.Liu, unpublished observations); furthermore, few receptors are expressed in Ltk- cells, including thrombin, purinergic P2 and prostaglandin E2 (PGE2) receptors (Liao et al, 1990; see Table 1).

The full length cDNAs of the rat or human 5-HT1A, D1 or D2 (long or short form) receptors were separately inserted into eucaryotic expression vector, PEM-3; the resulting constructs were stably expressed in GH4C1 or Ltk- cells respectively. The various GH4C1 and Ltk- subclones isolated are summarized in Table 2. Conveniently, the signalling patterns of endogenously expressed hormone receptors, including somatostatin, muscarinic-M4, TRH receptors in GH4C1 cells, and thrombin and purinergic-P2 receptors in Ltk- cells have served as internal controls for those of the transfected receptors.

Initially, the signal transduction pathways of the 5-HT1A, the dopamine-D1 and -D2 (short or long form) receptors were characterized in both GH4C1 pituitary and Ltk- fibroblast cells, and pretreatment with TPA or 8-Br-cAMP which was used to study the roles of PKA or PKC in modulation of the signalling pathways mediated by each receptors. To distinguish the role of a given PTX-sensitive G protein in specific receptor/effector interactions, distinct antisense G protein α subunit expression vectors were stably transfected into GH4C1 cells to deplete the gene expression of each individual α subunit; the antisense transfectants were utilized to dissect the roles each α subunit in the coupling of multiple receptors to particular intracellular signals.

Table 1. The differences between the rat GH4C1 pituitary and mouse Ltk- fibroblast cells

	Rat GH4C1 Pituitary cell	Mouse Ltk- Fibroblast Cell
Cell Growth	Very Slow	Fast
G Protein Distribution	αs, αq/11, αo1, αo2, αi1-3, α12, α13, ß1-4, γ2-7	αs, αq/11, αi2, αi3, ß1-3
Voltage-dependent Ion Channels	Calcium & Potassium Channels	None
Synthesis & Secretion of Hormones	Growth Hormone & Prolactin	None
Hormone Receptors	VIP, TRH, Muscarinic-M4, Somatostatin & galanin	ATP, PGE2, Thrombin

Table 2. Summary of stably-transfected cell lines subcloned from GH4C1 or Ltk- cells

Transfected Receptor	GH4C1	Ltk-
Dopamine D1 (human)	GH4-hD1	L-hD1
Long Form of Dopamine-D2 (human)	GH4D2L	LhD2L
Short Form of Dopamine-D2 (human)	GH4D2S	LhD2S
Short form of Dopamine-D2 (rat)	GH4ZR7	RGB-2
5-HT1A (rat)	GH4ZD10	LZD-7

CHAPTER II: RESEARCH BACKGROUND

Part I: Diversity of G Proteins

G proteins are termed by their common property of GTPase activity, and the ability to bind to GTP and hydrolyze GTP to GDP (Gilman, 1987). This property of hydrolyzing GTP is crucial to G protein function as a molecular switch for mediation of diverse cellular functions (Bourne et al, 1990; Kaziro et al, 1990). The mechanisms of G protein activation and inactivation are based on studies of translational factors, and are analogous mechanism of protein activation or inactivation by phosphorylation and dephosphorylation reactions (Bourne et al, 1990; Kaziro et al, 1991). G proteins undergo two alternate conformations: the GTP-bound form (active conformation) and the GDP-bound form. The GTP-bound form allows the protein to recognize and to interact with its target molecules; upon hydrolysis of GTP to GDP and inorganic phosphate, the protein is shifted to the GDP-bound form. The conversion of the GDP-bound form to the GTP-bound form is achieved by the exchange of the bound GDP with an external GTP. Each G protein α subunit has a characteristic dissociation constant for guanine nucleotides, Kd value for GTP and GDP, and the rate constant for GTP hydrolysis. Depending on the kinetic parameters of each G protein, factors that stimulate the exchange reaction, such as agonist-occupied receptors, or factors that increase the GTP hydrolysis rate, eg. GTPase-activating protein (GAP) modulate the state of G protein activation (Bourne et al, 1990; Kaziro et al, 1991).

G protein α subunits range from 39 to 52 kilodaltons in apparent molecular weight on SDS-PAGE. Molecular cloning techniques have provided powerful tools to identify the diversity

of G proteins. At present, many new G proteins have been identified in eucaryotic organisms (Simon et al, 1991). Heterotrimeric G proteins come from a large gene family, known to contain at least 16 different genes that encode α subunits, 4 that encode ß subunits, and multiple genes encoding γ subunits (Simon, et al 1991), resulting in over 300 possible heterotrimeric G proteins from different combinations of α subunits and ßγ dimers.

1. Stimulatory G proteins

Ross and Gilman analyzed the defect in the *cyc-* mutant of the S49 murine lymphoma cell line using reconstitution analysis, and they found that a GTP-binding component, defined as G/F, or N, promoted the coupling between the hormone-receptor complex and the activation of adenylyl cyclase (Ross & Gilman, 1977). The component of G/F or N is now designated as Gs.

The first full-length αs cDNA sequence, αs-1 encoding a 394-amino acid protein was cloned from a bovine adrenal cDNA library (Robishaw et al, 1986a). Transient expression of this αs cDNA in COS-7 cell led to expression of a 57K form of αs as measured by Western blot analysis (Robishaw et al, 1986a). A second form of αs, termed as αs-2 was subsequently isolated. The coding region of αs-2 is identical with the first one except there is a deletion of 42 nucleotides leading to loss of 14 amino acids, with two flanking amino acid changes (Robishaw et al, 1986b). αs-2 cDNA directs expression of a 43K protein of αs in COS-7 cell. The third form of αs, or αs-3, was isolated from the PU-5 murine macrophage cell line. The cDNA encodes a protein similar to the short form of αs but lacking a single serine residue at the point where a 14-amino acid insertion distinguishes the long and short forms of αs (Sullivan et al 1986). The fourth form of αs or αs-4, was cloned soon after from human brain which encodes an identical protein as the first one with the additional serine residue at the C-terminal

of the 14-amino acid insertion (Bray et al, 1986). All four forms of αs are derived from alternative splicing of a single gene transcript.

Human αs gene contains 13 exons and 12 introns and span about 29 kb (Kazosa et al, 1990). Exons 3 and 12 encode the unique translated sequence found for the four forms of αs at the N- and C- terminal regions, respectively; the four different αs cDNA's are generated by alternate use of exon 3 and/or of two 3' alternative splicing sites of intron 3 (Kazosa et al 1988). In comparison to αs-3, αs-1 lacks 45 nucleotides (15 amino acids) which are encoded by exon 3. αs-2 and αs-4 have additional nucleotides (CAG) to αs-1 and αs-3, respectively, 3' to the above 45 nucleotides (Kazosa et al, 1988).

The αs subunit RNA's are expressed in almost every tissue that has been examined (Simon et al, 1991). All four forms of αs are substrates for ADP-ribosylation by cholera toxin. Cholera toxin (CTX) contains ADP-ribosyltransferase activity which catalyze transfer of ADP-ribose from NAD+ to αs; ADP-ribosylation of αs stabilizes the GTP-bound conformation of αs by decreasing its intrinsic GTPase activity, resulting in constitutive activation of adenylyl cyclase (Cassel & Selinger, 1977; Cassel & Pfeuffer 1978). Since the ADP-ribosylation of αs by CTX is irreversible and the ADP-ribosylated αs is stable, activation of adenylyl cyclase by CTX is persistent (Cassel & Selinger, 1977; Gilman 1987).

Analysis of hormone-receptor complex interactions with Gs was performed by reconstitution of purified protein into phospholipid vesicles. It was shown that ß-adrenergic receptor complex was able to enhance the rate of GTPγS binding to Gs at low Mg++ concentrations (Asanol et al, 1984). The binding of GTPγS to Gs under these conditions occurred concomitant with an activation of Gs, as assessed by its ability to stimulate the cyc-

S49 cell membrane adenylyl cyclase (Asanol et al, 1984). In S49 cyc- lymphoma cells, which lack αs subunits, stable transfection of αs1 cDNA restored the ability of ß-adrenergic ligand to stimulate adenylyl cyclase (Nukada et al, 1987). Bacterially expressed αs1 or αs4 subunits stimulate adenylyl cyclase activity when added to S49 cyc- cell membranes, an action which is inhibited by addition of excess ßγ dimers (Graziano et al, 1989). Both 45-kDa and 52-kDa αs subunits stimulate adenylyl cyclase, although there is evidence that the 45-kDa αs has greater functional activity than the 52-kDa αs (Graziano et al, 1989; Walseth et al, 1989).

Varying in different tissues, activation of Gs coupled receptors can directly or indirectly mediate the increase of voltage-dependent calcium channel activity (Birnbaumer et al 1990). Studies by using reconstitution of purified calcium channels with pre-activated αs into liposomes demonstrated that Gs can directly open DPH-sensitive calcium channels in cardiac and skeletal muscle (Yatani et al, 1987; 1988; Hamilton et al, 1991). In neurons and endocrine cells, Gs indirectly mediates opening of voltage-dependent calcium channels via elevation of cAMP to activate PKA. In hippocampal neurons, cAMP elevation has been shown to enhance calcium influx (Gray & Johnson, 1987). In AtT-20 or GH4C1 pituitary cells, CRF or VIP receptors, via elevation of cAMP, potentiate the activation of calcium channels, an effect which is crucial for the CRF- or VIP-induced hormone secretion (Luini et al 1985; Guild et al, 1986; Enyeart et al 1990).

Structure-function analysis of αs has been carried out by using site-directed mutagenesis. The αs contains multiple function domains which are involved in GTP binding, GTP hydrolysis, ßγ association, receptor recognition, and effector interaction and regulation (Gilman, 1987; Kaziro et al, 1991; Berlot & Bourne, 1992). The mutation of Gly-49 impaired GTP hydrolysis

ability Gs (Graziamo & Gilman, 1989; Masters et al, 1989). Gln→Leu mutation at the position 227 reduced the intrinsic GTPase activity, and induced the constitutive activation of αs (Graziano & Gilman, 1989; Masters et al, 1989). The ADP-ribosylation site of αs is Arg-201 (Kaziro et al, 1991); mutation of this residue reduced the rate of GTP hydrolysis of αs subunits and caused constitutive activation of adenylyl cyclase (Landis et al, 1989; Masters et al 1989). The domains which interact with adenylyl cyclase are also localized near the C-terminus, site-directed mutagenesis of the amino acid residues, Gly-225, Trp-263, Leu-268, and Arg-269 resulted in partial or complete loss of the ability to stimulate adenylyl cyclase (Itoh & Gilman, 1991; Osawa & Johnson, 1991). The region which interacts with ßγ subunits is thought to be localized at the N-terminus; deletion of αs 2-29 impaired its interaction with ßγ dimers and made it insensitive to CTX (Journot et al, 1991).

Structurally and functionally, αolf resembles to αs and shares 88% amino acid sequence identity with αs (Kaziro et al, 1991; Simon et al, 1991). Unlike αs, αolf gene expression is restricted to olfactory epithelium, where it stimulates adenylyl cyclase (Jones and Reed, 1987). The site for the CTX-mediated ADP-ribosylation in αolf is identical to the site in αs (Jones & Reed, 1988)

2. Inhibitory G proteins

The inhibitory G proteins comprises 8 different α subunits, αi1, αi2, αi3, αo1, αo2, αz, and two transducins, αt1, αt2 (Simon et al 1991). Three different αi cDNAs have been isolated from a variety of tissues and are encoded by three separate genes (Itoh et al 1986; 1988; Jones and Reed 1987). They are highly homologous, and share 85% identity of amino acid sequences (Jones and Reed, 1987; Itoh et al, 1988; Kaziro et al, 1991; Simon et al, 1991); αi1

consists of three exons and two introns; the human αi2 and αi3 genes have eight exons and seven introns (Itoh et al 1988). The splice sites of the human αi1, αi2 and αi3 genes are almost identical, suggesting that they were from gene duplication of the same ancestor (Itoh et al, 1988).

The G protein αi subunits appear to have distinct tissue distributions: αi3, is expressed in all tissues examined; αi1 is mainly expressed in neurons; while αi2 is dominantly expressed in peripheral tissues, more weakly expressed in brain, and is not detectable in olfactory and liver (Jones and Reed, 1987, Itoh, et al, 1988; Brann et al, 1987).

The αo, which accounts for 0.5-1% of membrane-bound proteins of brain tissues, was purified from bovine brain free of associated ßγ dimers (Sternweis & Robishaw, 1984; Neer et al, 1984). The αo subunits share 73% identity with αi subunits and 34% (50% homology) identity with αs subunits. Two types of the αo cDNAs, αo1 and αo2, derived from alternative splicing of a single copy of the αo gene, were isolated from various species (Strathmann et al, 1990; Bertrand et al, 1990; Tsukamoto, et al, 1991). The human αo gene contains 11 exons. Exons 7 and 8 encoding for amino acid residues 242 to 354, are duplicated, and each pair of the duplicates encodes αo1 and αo2 respectively, the splicing sites of the αo gene are completely identical with those encoding αi2, αi3 and transducin, suggesting these genes are evolved from a common progenitor (Tsukamoto, et al, 1991).

The αo subunits are mainly expressed in neurons and endocrine cells (Asano et al 1987; Brann et al 1987). In bovine brain, αo subunits were found to be more abundant in cerebral cortex, thalamus, hypothalamus, and cerebellum (Asano et al, 1987; Brann et al 1987). Ultrastructural localization showed that αo subunits were largely localized to the plasma

membrane but were not detected in pre- or post-synaptic membranes (Gabrion et al, 1989). Interestingly, there is different subcellular distribution of αo subunits in neurons and in glia cells. In neurons, αo subunits were mainly localized in plasma membrane, while in glia cells, they were primarily detected in cytoplasmic matrix, suggesting the different roles of Go in neurons and glia cells (Brabet et al, 1988; Gabrion et al 1989).

Unlike αs, the N-terminus of αo or αi subunits is myristylated (Buss et al, 1987). Myristylation increases the affinity of α subunits for ßγ dimers, facilitates heterotrimeric association, stabilizes the interaction of α subunits with the phospholipase bilayer or with membrane-bound proteins, and allows α subunits attach directly to membrane (Spiegel et al, 1992). The N-terminus of αo is thought to contain domains required for the binding to the ßγ subunits (Robishaw et al, 1989; Denker et al, 1992). The C-terminus of αo/αi is the domain which links to receptors, and perhaps to effectors (Kaziro et al 1991; Spiegel, et al, 1992). The cysteine residue in the fourth position from the C-terminal (C351) is the site for ADP-ribosylation by PTX (West et al, 1985). This cysteine appears also to be critical for αo/αi to interact with hormone receptors (Kaziro et al, 1991; Spiegel et al, 1992).

Transducins include αt1 and αt2; structurally, they resemble αi and αo subunits. The αt subunits contain the domains for ADP-ribosylation by both CTX and PTX , thus they are sensitive to both CTX and PTX-mediated ADP-ribosylation (Reed 1992). The expression of αt subunits is restricted to retinal rod outer segments, where they mediate the coupling between rhodopsin and a cyclic GMP phosphodiesterase (Reed 1992).

The αz subunit bears some resemblance to αi subunits, but does not contain the C-terminal Cys residue required for modification by PTX. Functionally, it appears to be an unique

G protein α subunit. In comparison with the other α subunits, αz has very low intrinsic GTPase activity; the rate of GTP hydrolysis by αz is about 100 times slower than that by the other G protein α subunits (Wang et al, 1991; Kaziro et al 1991). In addition, αz has an unusual Mg++ ion dependence. The αz is primarily found in neurons, particularly in neurons with long axonal processes. The functions of Gz remain obscure. One study indicated that Gz may mediate inhibition of adenylyl cyclase via a PTX-insensitive pathway (Wang et al, 1991).

Except for αz, all other members of the αi class contains sites susceptible to be ADP-ribosylated by PTX. ADP-ribosylation by PTX causes the ADP-ribose to attach to the C-terminal cysteine of $\alpha i/\alpha o$ and prevents their specific interaction with hormone receptors (Ui, 1986); however, it does not alter intrinsic GTPase activity of the α subunits (Sunyer et al., 1989).

It was suggested that all Gi may be able to induce inhibition of adenylyl cyclase (Birnbaumer et al 1991). In platelets, the $\alpha 2$-adrenergic receptor-induced inhibition of adenylyl cyclase is mediated by Gi2 (Simonds et al 1990). Site-directed mutagenesis of the key amino acid residues at the consensus sequence NKXD of $\alpha i1$, $\alpha i2$, or $\alpha i3$ induced constitutive inhibition of adenylyl cyclase (Wang et al, 1991). The $\alpha i2$ mutants, termed *gip*-2 were isolated in ovary and adrenal cortical tumours, are the result of non-conservation amino acid substitutions of arginine at position 179 by cysteine ($\alpha i2$-R179C), or glutamine at position 205 by leucine (Lyons et al, 1990). The *gip*-2 is GTPase deficient, suggesting a stable and active conformation. Several studies have shown that *gip*-2 induced constitutive inhibition of forskolin-stimulated adenylyl cyclase activity, attenuated the thrombin receptor-stimulated phospholipase A2 activity, and constitutively activated MAP kinase in NIH 3T3 cells, CHO cells and rat-1 cells (Wang et

al, 1991; Lowndes et al, 1992; Gupta et al, 1992).

Numerous Gi/Go-coupled receptors, including 5-HT1A, α2-adrenergic, dopamine-D2, muscarinic-M4, $GABA_B$, somatostatin have been shown to induce closure of voltage-dependent calcium channels in neurons, neuronal cell lines, and endocrine cells (Spiegel et al, 1992 ref within). In dorsal root ganglia, activation of the α2-adrenergic or $GABA_B$ receptors initiated a PTX-sensitive inhibition of calcium current (Dolphin and Schott, 1989). In NG-108-15, opiate receptor activation has been shown to inhibit voltage-dependent calcium channels via specific PTX-sensitive G proteins (McFadzean et al, 1989).

The G protein involved in inhibition of calcium channels is Go. This conclusion is based on the following evidence. Injection of the purified αo or αi proteins into neurons restores the ability of hormone receptor-mediated inhibition of calcium current, and αo was ten times more potent than αi subunits (McFadzean et al, 1989). Injection of antibodies against αo but not αi subunits blocked the receptor-induced inhibition of calcium current (McFadzean, et al, 1989). Furthermore, introduction of the antisense oligonucleotides specific for αo1 or αo2 selectively blocked muscarinic-M4 or somatostatin receptor-mediated inhibition of calcium current (Kleuss et al, 1991).

The G protein which gates potassium channels is Gi3. Application of the highly purified αi3 to excised atrial patches myocytes (Yatani et al, 1987) cardiac cell (Codina et al, 1988), and GH4C1 pituitary cells (Yatani et al, 1987), or in skeletal muscle in lipid bilayer (Mattera et al, 1989) induces opening of potassium channels. It remains controversial at present whether Go also gates potassium channels. Introducing of the highly purified and proactivated αo initiated four different potassium currents in inside-out patches on hippocampal pyramidal neurons

(VanDongen et al, 1991). However, using similar approach, αo failed to gate muscarinic potassium channels in guinea pig atrium; αi1 and αi2 were able to gate potassium channels, but were less potent than αi3 (Yatani et al 1988; Brown and Birnbaumer, 1990).

Depending on the cell systems, different PTX-sensitive G proteins can inhibit or stimulate PLC activity. PTX pretreatment inhibited PI turnover in mast cells (Nakamura and Ui, 1985), and renal mesangial cells (Pfeilschifer and Bauer, 1986). In primary brain culture, pretreatment with PTX blocked α1-adrenergic receptor-mediated activation of PLC (Wilson and Minneman, 1990). PTX-sensitive G proteins has been also shown to stimulate phospholipase C activity. In HL-60 cells, fMLP receptors induced PTX-sensitive activation of PLC (Kikuchi et al, 1986). Transfection of α2C-adrenergic receptor in Rat-1 cells (Milligan et al 1991), 5-HT1A receptor in COS-7 and Hela cells (Fargin et al, 1989; Raymond et al, 1991), and the D2S receptor in Ltk- cells induces PTX-sensitive activation of PLC.

The G proteins which mediate stimulation of PTX-sensitive activation of PLC remain to be established. Injection into Xenopus oocytes of αo subunits but not αi subbunits, evoked a calcium-dependent Cl- currents; the currents were mediated by calcium mobilization from IP3-sensitive calcium stores in oocytes, suggesting that Go mediates PLC activation to increase IP3 levels in oocytes (Moriarty et al, 1990). Go was also shown to enhance muscarinic-M2 receptor-stimulated Cl-current in oocytes, further supporting the role of Go in activation of phospholipase C activity (Moriarty et al, 1990). However, αo, αi, and αz failed to stimulate PLC-ß in vivo (Taylor et al 1991; Waldo et al 1991). Recent evidence indicates that the action of fMLP receptors on PLC may be mediated by ßγ dimers (Camps et al, 1992a & 1992b; Birnbaumer 1993).

PTX-sensitive G proteins have been shown to directly and indirectly stimulate phospholipase A2. A direct link between G protein and phospholipase A2 was suggested by the finding that PTX reduced the calcium-mediated GTPγS-dependent release of arachidonic acid in FRTL-5 and mast cells as well as neutrophils (Murayama et al, 1985). In permeabilized mast cells, agonist-induced stimulation of phospholipase A2 was mimicked by GTPγS, and was blocked by pretreatment with PTX (Burch et al, 1986). In CHO cells, activation of the transfected dopamine-D2S or α2-adrenergic receptors did not stimulate phospholipase A2, but significantly enhanced ATP-stimulated arachidonic acid release. This action of D2 or α2 receptors on phospholipase A2 was blocked by PTX (Felder et al, 1992). It is not known which PTX-sensitive G protein is involved in mediation of phospholipase A2 activity. In CHO and NIH 3T3 fibroblast cells, constitutively active *gip2* has been shown to attenuate phospholipase A2 activity (Lowndes et al, 1991).

3. Gq and phospholipase C

Gp or Gq, designated as an unique class of G proteins, regulates PTX-insensitive activation of phospholipase C activity. Molecular cloning has revealed that the αq class includes at least five structurally-related members, αq, α11, α14, α15 and α16. The α11 resembles αq, the deduced amino acid sequences of α11 and αq are 88% identical. Both α11 and αq are widely-distributed. While α14 is mainly expressed in stromal and epithelial cells, α15 and α16 are detected in the cells derived from the haematopoietic lineage, and myeloid cells. In addition, α15 is found in murine B lymphocytes and α16 is present in human T lymphocytes (Simon et al 1991). The αq class does not contain sites for ADP-ribosylation by PTX or CTX, and are insensitive to both CTX and PTX (Blank et al, 1991).

PLC is the key enzyme to regulate cellular metabolism (Berridge & Irvine 1989; Berridge 1993). Activation of PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to yield IP3 and DAG. IP3 induces the release of intracellular calcium stores, whereas DAG is the physiological activator of PKC (Berridge & Irvine, 1989; Berridge, 1993). Purification and molecular cloning have confirmed the existence of at least 16 isoforms of mammalian phospholipase C (Rhee & Choi, 1992). On the basis of the sizes and structural similarities, phospholipase C have been divided into three principal classes, namely, PLC-ß, PLC-γ and PLC-δ. The PLC-ß activity is regulated by G proteins, whereas the PLC-γ activity is regulated by receptors of the tyrosine kinase family (Rhee & Choi, 1992). The regulators for PLC-δ remain to be determined.

Numerous studies have indicated that the Gq class is involved in PTX-insensitive receptor mediated activation of PLC (Park et al, 1992a; Rhee et al, 1992). PLC-ß is the specific effector involved in the Gq class-mediated activation of PLC. The purified αq has been shown to induce activation of PLC in the presence of AlF^-4 and ßγ dimers (Blank et al, 1991). When reconstituted in the presence of GTPγS with different isoforms of PLC, the mixture of αq and α11 specifically stimulated PLC-ß1 but not PLC-δ1 or PLC-γ1 (Wu et al, 1992a). Co-transfection of PLC-ß and αq or α11 cDNAs markedly increased basal level of inositol phosphate formation in COS-7 cells (Wu et al, 1992). The introduction of a mutation of (Gln-209→Leu) caused constitutive activation of αq and α11 and resulted in a persistent activation of PLC activity and high basal levels of inositol phosphates (Wu et al, 1992b).

The relative selectivity of the αq class in activation of PLC-ß isoenzymes has been determined. Reconstitution of the membranes from COS-7 cells transfected with αq, α11, α14,

or α16 indicated that they all can stimulate PLC-ß1 (Wu el al, 1992), α16 was found to activate more efficiently to PLC-ß2, αq, α11, α14 showed less effective stimulation (Wu et al 1992b).

4. G protein ßγ dimers

In most tissues, the ß subunit of G proteins is a mixture of 35-kDa and 36-kDa proteins (Neer et al, 1989). Four different ß subunit cDNAs have been cloned (Simon et al 1991). They share 80% of amino acid sequence identity (Simon et al, 1991). The ß1, ß2, and ß3 subunits are widely expressed, while the ß4 subunit is strictly expressed in lung and brain tissue and has a very low level expression in the other tissue (Fung et al, 1987; Gao et al, 1987; Simon et al, 1991). All ß subunits are composed of eight repeated segments of 40-amino acids each that contains characteristic motifs, in particular a tryptophan-aspartic acid paries. This motif is referred as the WD-40 repeat which has been found in a variety of functionally unrelated proteins, and as yet its functional significance remains unknown.

The γ subunits are roughly 70 amino acids long, and vary in apparent size from 5-10 kDa on SDS-PAGE (Simon et al, 1991). Five different genes encoded γ subunits have been isolated (Gautum et al, 1990; Robishaw et al, 1989). Peptide sequences obtained from purified γ subunits suggest that there are at least two more γ subunit isoforms in mammalian tissues. The γ1 subunit is mainly expressed in retina, while the γ2 subunit is widely expressed in almost all tissues. The γ3 subunit is restricted to the brain and testis. Comparison of the amino acid sequences of γ1 (or γt) subunit from retina with the four other γ subunits from nonretinal sources showed significant variability; the γ1 and γ2 sequences differ in 44 of 71 amino acid residues with the greater number of differences occurring in the N-terminal (Gautum et al 1990). Functionally, the γ1 subunit is significantly different from the other four γ subunits. All γ

subunits share considerable sequence homology at their C-terminus (Simon et al 1991), they all have a C-terminal C-A-A-X motif, which undergoes three sequential post-translational modifications, isoprenylation, and then modification by adding farnesyl or geranylgeranyl moiety depending on the last amino acid of the γ subunit (Spiegel et al, 1991). Modifications of the C-terminus of the γ subunits are crucial for membrane attachment of $\beta\gamma$ dimers (Spiegel, 1991).

Various combinations of the ß and the γ subunits are associated with different α subunits giving the potential for a large number of G protein isoforms. Given at least four different ß subunits, and seven different γ subunits, despite their tissue-specific distribution, the possible numbers of combinations of these subunits into the distinct $\beta\gamma$ dimers is relatively large (4ß X $7\gamma = 28\beta\gamma$), although some of these combinations may not naturally exist (Pronin & Gautam, 1992). Of interest is whether a specific $\beta\gamma$ dimer is required to interact with a specific α subunit to modify its function. The $\beta\gamma$ dimers from different sources are functionally interchangeable, although there is marked difference between the coupling of $\beta 1\gamma 1$ and any other type of $\beta\gamma$ subunits to $G\alpha s$ and $G\alpha t$ (Cerione et al, 1987) and the $\beta\gamma$ dimers from retina were less effective than a mixture of the $\beta\gamma$ dimers from brain in interacting with $G\alpha s$ to inhibit adenylyl cyclase activity (Cerione et al, 1987). Theoretically, the 28 possible combination of distinct $\beta\gamma$ dimers will generate over 400 (16α X 28 $\beta\gamma$) possible G protein heterotrimeric combinations, including 28 Gi1, 56 Go, and over 100 different Gs forms.

The early studies showed that increasing the free $\beta\gamma$ concentration reversed the activation of adenylyl cyclase by GTPγS-ligand αs (Northup et al 1980). This finding led to the prediction that G protein $\beta\gamma$ dimers modulate the function of α subunits. But this appears not to be the case for G protein-mediated mating responses in yeast where $\beta\gamma$ dimers are responsible for

activating the mating pathways and α subunits apparently prevent signalling by $\beta\gamma$ dimers in the absence of the receptor/ligand interaction. Null mutations of the genes encoding G protein α subunit (GPA1), ß subunit (STE4) or γ subunit (STE18) in yeast demonstrate that both genes encoding Gß and Gγ are required for mating responses, mutants defective in these genes are unresponsive to the mating factors (Blumer & Thorner 1990). Null mutation of the Gα gene results in constitutive activation of all mating pheromone response, presumably because an activator of the response pathway is liberated (Blumer & Thorner 1991). But so far, no downstream effector has been identified to link G$\beta\gamma$ dimer in the mating response in yeast.

In the mammalian systems, G protein $\beta\gamma$ dimers have been shown to suppress α-GDP dissociation to maintain α subunits in their inactive state (Gilman, 1987). They are required for the high affinity state of hormone receptors and perhaps, the specificity of receptor coupling to G proteins (Journot et al, 1991; Kleuss et al, 1992a, 1992b). Recent studies indicate that thay may also directly regulate certain effector activity, including types II and IV adenylyl cyclase (Tang & Gilman 1991; Federman et al, 1992, PLC-ß, phospholipase A2 (Kim et al, 1989; Axelrod et al, 1988; Jelsema & Axelrod, 1987) and cardiac atrial potassium channels (Logothetis et al, 1987); and to be involved in desensitization of G protein coupled receptor signalling (Pitcher et al, 1992).

Several studies have shown that $\beta\gamma$ dimers can mediate adenylyl cyclase activity. Deletion of N-terminus of αs not only impaired the ability of αs to associate with the $\beta\gamma$ dimer but also disrupted Gs-mediated activation of adenylyl cyclase, and decreased the agonist binding affinity of ß-adrenergic receptor, suggesting that $\beta\gamma$ dimers are required by Gs coupled receptor-mediated stimulation of adenylyl cyclase (Journot et al, 1991). To date, eight different types

of adenylyl cyclase have been discovered; they all can be activated by αs (Tang & Gilman, 1992a). It is striking that, while ßγ dimers inhibit type I adenylyl cyclase, they stimulate types II and IV adenylyl cyclase, and have no effect on the other types of adenylyl cyclase, eg. types III, V, VI (Tang & Gilman, 1992a & 1992b). Stimulation of adenylyl cyclase by ßγ dimer is conditional because it requires pre-activation of adenylyl cyclase by αs (Tang & Gilman, 1992a). In intact cells, ßγ dimers have been also shown to induce stimulation of type II adenylyl cyclase. In 293 kidney cells, the Gs-mediated activation of adenylyl cyclase was inhibited by the dopamine-D2S or adenosine receptors. When type II adenylyl cyclase was transfected into 293 cells, both D2S and adenosine receptors switched to enhance Gs-mediated activation of adenylyl cyclase (Federman et al, 1992).

In HL-60 cells, fMLP receptors stimulate PLC activity via a PTX-sensitive G protein (Kikuchi et al 1986). Recent studies indicated that this action may be mediated by free ßγ dimers. ßγ dimers purified from retina stimulated an unidentified PLC in soluble fraction of cultured HL-60 cells. Both recombinant PLC-ß1 and PLC-ß2 but not PLC-δ are shown to be activated by ßγt dimers, PLC-ß2 is more sensitive (Camps et al, 1992). The ßγ dimers purified from bovine brain have been shown to differentially stimulate purified PLC isoenzymes in the reconstituted lipid vesicles in the order of PLC-ß3 > PLC-ß2 > PLC-ß1 > PLC-δ (Park et al, 1993). Interestingly, stimulation of PLC-ß by ßγ dimers required 1,000-fold higher concentration than that for αq subunits (Camps et al, 1992; Park et al, 1993). Stimulation of PLC by ßγ dimers has been also shown in intact cells. In COS-7 cells, stimulation of the transfected muscarinic-M2 receptor induced a small increase PI turnover; this action is markedly enhanced when ßγ dimer or PLC-ß2 cDNAs were co-transfected (Katz et al, 1992). These

studies demonstrate that free ßγ dimers can stimulate PLC-ß in either reconstituted system or in intact cells. Since the levels of αi and the density of fMLP receptors are very high in HL-60 cells, it was suggested that it was ßγ dimers rather than α subunits mediated PTX-sensitive activation of PLC (Birnbaumer 1992).

In the rod outer segments (ROS) of retina, addition of transducin induced activation of cGMP phosphodiesterase and phospholipase A2 (Bourne & Stryer, 1986). Activation of cGMP phosphodiesterase is mediated by an α subunit; in contrast, stimulation of phospholipase A2 is mediated by free ßγ dimers (Jelsema & Axelrod, 1987). The αt subunit, which itself slightly stimulated phospholipase A2, inhibited ßγ dimer-mediated activation of the enzyme (Jelsema & Axelrod, 1987). Activation of cardiac muscarinic receptors induced opening of potassium channels, this action can be direct or secondary to activation of phospholipase A2 and is mediated by free ßγ dimers (Logothetis et al, 1987; Kim et al, 1989).

Injection of ß or γ antisense oligonucleotides into GH3 pituitary cells demonstrated that the presence of distinct ß and γ subunits is required for receptor-mediated inhibition of calcium currents (Kleuss et al, 1992, 1993). Injection of antisense oligonucleotides common to four cloned ß subunit cDNAs completely blocked muscarinic-M4 and somatostatin receptor-mediated inhibition of calcium currents in GH3 cells (Kleuss et al, 1991). Further studies using specific antisense oligonucleotides have shown that muscarinic-M4 receptor-mediated inhibition of calcium current was specifically blocked by ß3 or γ4 antisense oligonucleotides while the action of somatostatin was abolished by ß1 or γ3 antisense oligonucleotides (Kleuss et al, 1992, 1993). These studies indicated that a receptor can select among many possible ßγ dimer combinations. Since the selected ßγ dimers by a receptor may be specific to the receptor and perhaps even to

a receptor-mediated signal, they may contribute to the specificity of the cellular responses that a receptor elicits. Furthermore, receptor-specific kinase, ßARK, has been shown to bind to ßγ dimers, suggesting that ßγ dimers may also involved receptor desensitization (see Part III)

PART II. The Specificity of G Protein in Coupling of Hormone Receptor and Effectors

The common structural feature of seven transmembrane domains allows receptors to associate with G proteins, while variations in their intracellular cytoplasmic loops limit receptor interactions with a subset of G proteins (Dohlman et al, 1991; Kobilka et al, 1992). According to the subset of G proteins activated, G protein coupled receptors can be classified into three major classes, Gs-, Gi/Go-, and Gq-coupled receptors. All G protein coupled receptors bear considerable amino acid sequence similarity, particularly at the transmembrane regions (Dohlman et al, 1991; Kobilka 1992).

Analysis of the functions of deletion mutants and chimeric receptors, the effects of peptides corresponding to portions of receptor sequence, and antibodies directed against different cytoplasmic domains has been used to map potential sites which participate in receptor coupling to G proteins. It has become evident that the concerted participation of several intracellular receptor domains is involved in coupling to G proteins (Dohlman et al, 1991; Kobilka 1992). These domains include the second cytoplasmic loop, third cytoplasmic loop, in particular the portions adjacent to transmembrane domains V and VI, and the N-terminal portion of the cytoplasmic tail. Portions of intracellular loops of receptor are important both in determining its affinity agonist and the specificity of coupling to G proteins and cellular signals to be produced (Dohlman et al, 1991; Kobilka 1992).

The N-terminal portion of the third cytoplasmic loop is a major determinant of selectivity in the receptor-G protein interaction; other domains including the second loop, and the C-terminal tail may play synergistic role for the receptor to interact with distinct G proteins (Dohlman et al, 1991; Kobilka, 1992). A chimera was structured in which the third cytoplasmic

loop of Gs-coupled ß2-adrenergic receptor was replaced with corresponding sequence from the Gq-coupled α1-adrenergic or muscarinic-M1 receptors: ß-adrenergic agonist binding activity of the chimeric receptors remained unaltered, however the chimeric receptor was coupled to stimulation of both adenylyl cyclase (ß-adrenergic phenotype) and PLC (α1-adrenergic phenotype, ref. Cotecchia et al, 1992; Wang et al, 1990). Thus the N-terminal portion of the third loop appears to be the major determinant for both M1 and α1 receptors to link to activation of phospholipase C. Similarly, replacement of the third loop of the M2-muscarinic receptor by M1 receptor sequences converted the receptor to stimulation of PI turnover (M1 receptor phenotype, ref. Cotecchia et al, 1992; Wang et al, 1990). Chimeras constructed from α2- and ß2-adrenergic receptors showed the third loop to have a dominant role in determining selectivity of the receptor coupling to Gs or Gi/Go (Kobilka et al 1988; Liggett et al, 1991). Site-directed or deletional mutagenesis defined that the amino acid residues of the third cytoplasmic loop, particularly at the juxtaposition to transmembrane-segment V and VI, are important in determining G protein coupling and specificity (Dohlman et al, 1991; Kobilka 1992). Mutations of residues located proximal to TM VI of the third loop caused constitutive activation of the receptor, on the other hand, mutation of residues at the N-terminus impaired receptor coupling to G proteins and inhibited receptor transmembrane signalling (Cotecchia et al 1990; Kjelsberg et al 1992). Synthetic peptides corresponding to the second and third cytoplasmic loops, or the C-terminal tail of rhodopsin, indicated that all three regions participate in receptor interaction with G proteins (Okamoto et al, 1991). The peptides corresponding to the second and the third loops of ß2-adrenergic receptor inhibited G protein coupling, whereas the peptide corresponding to the C-terminal portion of the third loop induced activation of adenylyl cyclase in the manner

of the agonist-occupied receptors (Okamoto et al 1991). Taken together, these results suggest that multiple intracellular domains of receptors might form a three-dimensional binding site allowing the receptor to recognize specific G proteins. An unique and interesting phenomenon from the construction of chimeric receptors is that despite the limited homology of hydrophilic domains between G protein coupled receptor family (eg. ß-, α1- or α2-adrenergic, 5-HT1 receptor family etc.), no obvious consensus sequence has been identified for any of these three major classes of the receptors for them to couple to Gs, Gq or Gi/Go. As yet, no unique sequence from different subtypes of receptors with similar pharmacological properties (eg. ß1, ß2-adrenergic receptors, M1, M3, M5 muscarinic receptors) has been identified to activate a specific G protein. One speculation is that the functionally similar receptors might activate a specific G protein via distinct sequences which share common structural features (Cotecchia et al, 1992).

A given cell may express multiple 7-TMS receptors, G proteins and a variety of effectors. Although determination of the specific G protein in a single receptor/effector interaction has been the subject of intensive investigation, unequivocal assignment of one G protein to a single receptor/effector system has been achieved only for transducin activation of cyclic GMP phosphodiesterase by photoreceptors (Stryer & Bourne, 1986). For many other receptors, however, the particular G protein involved in specific receptor/effector interactions remains to be explicitly identified.

Several approaches, including reconstitution of purified receptors and G proteins in liposomes, transfection or co-transfection of the cloned receptor and α subunit cDNAs, selective immunoprecipitation of receptor or G protein α subunits have been used to attempt to define the

specificity of hormone receptor coupling to distinctive G proteins.

The most common approach to determine which G protein is coupled to a given receptor/effector system is by reconstitution of receptor function using purified or recombinant G proteins. The vehicle for reconstitution experiments is usually artificial phospholipid vesicles, but it is also possible to reconstitute in a natural membrane environment. However, this approach cannot definitely show the specific G protein which links to an individual receptor/effector system *in situ* because purified receptors often are a mixture of different proteins. Some investigators have begun using recombinant receptors and α subunits purified from *E coli*; however, *E.coli*-derived α subunits are functionally less active and have low affinity for $\beta\gamma$ dimer than purified mammalian α subunits, presumably because of inadequate post-translation processing (Linder et al, 1991).

Based on the observations that the C-terminus of Gα subunits is involved in coupling to the receptor, immunoprecipitation of the soluble receptor using specific antibodies against the C-terminus of various Gα subunits has been utilized to determine the specificity of receptor to couple to individual G proteins. However, this approach is also limited since these antibodies have a low affinity and specificity to native G proteins (Spiegel et al 1992). Injection of antisense oligonucleotides specific to distinct G protein α subunits into cells provides a powerful tool to study individual G protein in receptor/effector interactions in single cell system, but this approach is only effective in a single cell preparations for electrophysiological measurement.

Of interest are the Gi/Go coupled receptor family which mediates multiple signal transduction pathways by coupling to one or more PTX-sensitive G proteins. Among Gi/Go coupled receptors, a particular G protein α subunit in the somatostatin receptor/effector

interaction has been extensively studied. Gαi3 can efficiently reconstitute somatostatin receptor-induced opening of potassium channels in GH3 pituitary cells (Yatani et al 1988). Following treatment with antibodies against various G protein α subunits, the binding activity of soluble brain somatostatin receptor was co-immunoprecipitated with αi1, αi2, or αi3 but not αo and αz (Murray-Whelan & Schlegel, 1989). Using the similar approach, another group showed that the solubilized somatostatin receptors were immunoprecipitated by antibodies against Giα1, Giα3, Gαo, Gß36, Gγ2 and Gγ3, but not by the antibody against Giα2 (Law et al, 1991). Studies using injection of distinct antisense oligonucleotides specific for different α, ß or γ subunit RNAs shown that somatostatin receptor-mediated inhibition of calcium currents was through αo2ß1γ3 heterotrimer, while the action of muscarinic-M4 receptor was through αo1ß3γ4 heterotrimer (Kleuss et al, 1991, 1992 & 1993). Apparently, there are clear contradictions of the specificity of somatostatin receptor coupling to G proteins from above studies; these differences may be due to variations of different approaches or the sources of the purified receptor and/or G proteins.

Stable transfection of receptor cDNA into cells with a particular complement of endogenous G proteins has been used to assess specific receptor/G protein interactions. This approach has been broadened by co-transfection of receptor and the α subunit. Since the interaction between receptor and G proteins may be affected by signalling environment which certainly varies in different cell systems, the results should be cautiously interpreted. Expression of three different α2-adrenergic receptor subtypes, α2B, α2C, and α2D in NIH-3T3 cells which express predominantly Giα2 and Giα3 showed that either α2B or α2D receptor required the presence of Gi2 to inhibit cAMP synthesis, whereas α2C required Gαo (Duzic et al 1992;

Coupry et al, 1992). Photolabelling of G protein α subunits using [α-32P]GTP azidoanilide showed that δ-opiod receptor from NG108-15 neuroblastoma-glioma hybrid cell coupled to Go and Gi2 but not Gi3 (Offermans et al 1991).

Nevertheless, despite some contradictions, current studies indicate that all PTX-sensitive G protein coupled receptors examined can interact with more than one, if not all, G protein α subunits. To date, a few hundred 7-TMS receptors have been cloned, none of them has the identical cytoplasmic domains. The foundation of specific receptor/effector interaction provided by G proteins remains to be addressed.

PART III Receptor Desensitization and Protein Kinases

The phosphorylation cycle includes three components: the substrate for phosphorylation; the protein kinase or phosphatransferase; and the phosphatase which catalyzes dephosphorylation of the phosphorylated substrate (Edelman et al, 1987). Activation of protein kinase is a major pathway known to regulate the rate of cellular protein phosphorylation (Edelman et al., 1987). Phosphorylation induced by protein kinases A and C or by ßARK, involves the reversible covalent modification of hydroxyl groups of serine and threonine residues in the substrate protein by a phosphotransferase reaction (Edelman et al, 1987). Protein kinase catalyzes the transfer of phosphate from ATP to substrate proteins on the specific serine and threonine residues (Edelman et al, 1987). Phosphorylation induces conformation change of the protein and thereby alters its function.

Phosphorylation attenuates receptor-mediated cellular responses, desensitizing transmembrane signalling of G protein coupled receptors. At least three major classes of serine/threonine kinases, including cAMP-dependent protein kinase, or protein kinase A, protein kinase C, and receptor-specific kinase (eg. ß-adrenergic receptor kinase (ßARK), rhodopsin receptor kinase (RK)) are involved in this process (Dohlman et al, 1991; Kobilka 1992). Activation of various effectors changes the levels of intracellular second messengers (eg. cAMP, Ca++ or DAG) which in turn stimulate PKA or PKC (Benovic & Lefkowitz, 1987). On the other hand, ßARK only recognizes the agonist-occupied ß-adrenergic receptors (Benovic et al 1989, 1990). Protein kinase, as a part of hormone receptor transmembrane signalling network, acts as a dual-function signal transducer, transducing and further amplifying intracellular signals (eg. cAMP, DAG); simultaneously, negative- or positive-modulating signal receptor signalling

(Lefkowitz et al 1990). Since hormone receptors, G proteins, effector enzymes, and ion channels are possible substrates of various protein kinases, modulation of receptor signalling system by kinases occur at multiple sites.

Protein Kinase A:

PKA is a cAMP-dependent protein kinase. The catalytic subunit of protein kinase A contains ATP-Mg binding site at its N-terminal which is rich in glycine (Uhler et al 1986; Showers & Maurer 1986). The regulatory subunit contains two cAMP binding sites A and B; binding of cAMP to site B renders the conformation of the regulatory subunit to make site A more accessible to cAMP. The regulatory subunit binds and inactivates the catalytic subunit, binding of cAMP to site A is required for the regulatory subunit to dissociate from the catalytic subunit (Uhler et al 1987).

Many synthetic cAMP analogues, such as 8-Br-cAMP, can activate protein kinase A. In contrast to cAMP, synthetic cAMP analogues are resistant to phosphatidyl esterase and they induce persistent activation of protein kinase A. Several lines of evidence indicate that phosphorylation by protein kinases regulates heterologous desensitization of Gs-coupled receptors (Dohlman et al, 1991). It may also be partially involved in desensitization of the Gi/Go or Gq class receptors (Liggett et al, 1992).

Protein Kinase C:

PKC is a calcium- and phospholipid-dependent protein kinase (Nishizuka 1988). The three major subspecies of protein kinase C, α, ß, and γ, accounting for approximately 95% of the protein kinase C proteins, have been cloned. Southern blot analysis has suggested that an even larger number of protein kinase C genes may exist (Coussens et al, 1987; Housey et al,

1987). Calcium and DAG are the major activators for PKC. DAG greatly increases the affinity of protein kinase C for calcium resulting in activation of this kinase at physiological concentrations of calcium (Elderman et al, 1987). Increase in intracellular free Ca^{++} alone does not induce activation of PKC. However, the enzyme can be activated by the synergistic action of an increase in $[Ca^{++}]i$ and the formation of DAG by receptor-mediated activation of PLC (Rhee et al 1992). TPA binds to the DAG binding site on PKC with higher affinity and dramatically increases the affinity of calcium for the protein kinase and activates PKC, both in vivo and in vitro (Castagna et al 1982).

It is clear that activation of PKC limits the duration and efficacy of the Gq-mediated activation of PLC in certain cell systems, including 3T3 fibroblast, CHO cells (Rhee & Choi, 1991). In most of cases, protein kinase C activation enhances the Gs-stimulated cAMP synthesis and suppresses the Gi-inhibited cAMP synthesis (Lefkowitz et al, 1990). It is clear now that PKC can directly stimulate basal and Gs-stimulated Type II adenylyl cyclase while having no effect on other types of adenylyl cyclase (eg. types I, III, IV, V and VI) in S49 or 293 cells (Jacobowitz et al 1993; Yoshimura & Cooper 1992). These results suggested that receptors which stimulate PKC may activate adenylyl cyclase independent of Gs.

ß-Adrenergic Receptor Kinase:

The ß-adrenergic receptor kinase, analogous to rhodopsin receptor kinase in a number of aspects, specifically induces phosphorylation of the agonist-occupied form of the ß-adrenergic receptor, and closely-related receptors (Benovic et al, 1990). Two distinct ßRK cDNAs have been isolated (Benovic et al 1990). ßRK1 cDNA encodes 689 amino acids. Analysis of the overall topology of ßRK1 reveals that it contains a N-terminal domain of 197 amino acids and

a C-terminal domain of 253 amino acids and a central catalytic domain which bears great sequence homology with PKA and PKC at the catalytic domains (33% identity for PKA and 34% for PKC); both N- and C-terminal domains lack significant homology to any other sequenced proteins (Benovic et al, 1989; 1990); these domains are thought to determine the specificity of the kinase to phosphorylate only agonist-occupied receptors (Benovic et al, 1990). The N-terminus of the catalytic domain has a Gly-X-Gly-X-X-Gly-$(X)^{10-16}$-Lys stretch which is present in all ATP-binding proteins; the C-terminus has an Asp-Leu-Gly consensus sequence, which is analogous to the sequence Asp-Phe-Gly in PKA and PKC, and appears to be involved in ATP binding (Benovic et al, 1990). Southern blot analysis indicated the presence of multiple genes encoding ßRK (Benovic et al, 1990).

In vitro, purified preparations of ßRK phosphorylate the agonist-occupied several G protein coupled receptors including ß2-adrenergic receptor (Benovic et al, 1989), α2-adrenergic receptor (Leeb-Lundberg et al, 1987), and muscarinic receptors (Kwatra & Hosey 1986). Expression of ßARK in COS-7 cells showed ßRK specifically induced phosphorylation of the agonist-occupied ß-adrenergic receptor, and had little effect on the light-bleached rhodopsin receptor (Benovic et al 1987).

Receptor Desensitization by Protein Kinases:

Desensitization is a general property of signalling systems which prevents prolonged stimulation. In Dictyostelium discoideum, the adaption of cAMP receptors allows the cAMP pulses to occur (Firtel et al, 1991). In the olfactory system, odorant signalling is terminated in the millisecond time range to ensure the system can be repetitively stimulated (Boekhoff & Breer 1992). Similarly, desensitization to hormone is a major factor limiting the efficacy and duration

of hormone actions.

Receptor desensitization can be divided into two distinct processes: the short-term termination of signalling, and the long-term reduced responsiveness despite the continuous presence of stimulus which is not going to be discussed here. In principle, desensitization could occur at any part of signal transduction pathway, receptor, G protein or effector, but it often occurs at the level of receptor itself (Lefkowitz et al 1990). The biochemical mechanisms of desensitization have been extensively characterized for ß-adrenergic receptors (Benovic et al 1990). Two distinct mechanisms underlie short-term desensitization: homologous desensitization, which leads to reduced responsiveness to the original stimulus; and heterologous desensitization, which leads to a decrease in responsiveness as the result of stimuli acting through other receptors (Lefkowitz et al, 1990; Dohlman et al, 1991).

In many systems, activation of PKA serves as an negative-feedback pathway to suppress Gs-mediated stimulation of adenylyl cyclase (Lefkowitz et al, 1990; Dohlman et al, 1991; Kobilka 1992). Activation of PKA is involved in the heterologous desensitization of the ß-adrenergic receptor (Bouvier et al, 1989a). By reconstitution of purified ß-adrenergic receptor with Gs, PKA has been shown to phosphorylate ß-adrenergic receptors and attenuate coupling to Gs (Bouvier et al 1989b). The action of PKA can occur when low concentrations of agonist are used; the phosphorylation of the receptor by PKA was slightly accelerated upon receptor activation by agonist (Bouvier et al 1989b). Two consensus sequences in the deduced sequence of ß2-adrenergic receptor have been shown to be phosphorylated by PKA: one is at the third cytoplasmic loop, the other is at the C-terminus (Hausdorff et al 1989). Further evidence for the involvement of protein kinase A in uncoupling of ß2-adrenergic receptor with Gs was

obtained using the synthetic peptide corresponding to the C-terminus of the third loop of the receptor (Okamono et al, 1991). This synthetic peptide induced activation of adenylyl cyclase from S49 lymphoma membranes via Gs; phosphorylation of this peptide by protein kinase A drastically reduced its effect on Gs and enhanced its effect on Gi (Okamono et al 1991).

The action of PKC varies in different cell systems. In L cells, phosphorylation of ß-adrenergic receptor by protein kinase C enhances receptor-mediated stimulation of adenylyl cyclase; mutagenesis to eliminate the potential phosphorylation sites for protein kinase C at the third loop of the receptor abolish this action of PKC (Yoshimasa et al 1987).

ßARK mediates homologous receptor desensitization (Benovic et al 1990). Like PKA, ßARK also induced the phosphorylation of ß-adrenergic receptor to uncouple the receptor from Gs (Benovic et al, 1990). Unlike PKA, the action of ßARK occurs primarily when a high concentration of agonist is applied, since the kinase phosphorylates only agonist-occupied receptors (Benovic et al, 1990). Deletion and site-directed mutagenesis experiments indicate that phosphorylation sites for ßARK are localized at the C-terminus of ß2-adrenergic receptor (Hausdorff et al 1989). Unlike rhodopsin kinase, which attaches to membrane by isoprenylation of the Cys-A-A-X motif at its N-terminus, ßARK links to the membrane by association with the G protein ßγ dimers (Pitcher et al 1992). Addition of the ßγ dimers to membrane preparations is required for the ßARK-induced phosphorylation of ß2-adrenergic receptor, suggesting that ßARK may uncouple ß-adrenergic receptor and Gs by binding to ßγ dimers (Pitcher et al 1992).

For most Gi/Go coupled receptors, little is known about the biochemical mechanisms of their desensitization. In most cases, prior activation of protein kinase A by forskolin or 8-Br-cAMP does not significantly affect receptor-mediated inhibition of adenylyl cyclase; for example,

in Ltk- fibroblast cells, D2S receptor-mediated inhibition of adenylyl cyclase remained unaltered after 30 min or 24h pretreatment with 8-Br-cAMP or forskolin, suggesting that protein kinase A may be not involved in heterologous desensitization of the D2S receptor-mediated inhibition of adenylyl cyclase (Bates et al, 1990). However, an in vitro study indicated that PKA can phosphorylate purified bovine striatal D2 receptors; this phosphorylation resulted in reduction of the affinity of the D2 receptor for agonist but not antagonist (Elazar & Fuchs, 1991). The α2A-adrenergic receptor-mediated inhibition of cAMP synthesis was significantly reduced after the receptor was exposed to epinephrine for 30 min; agonist-promoted desensitization was found to be accompanied by phosphorylation of the receptor in vivo, and can be removed by deletion all potential phosphorylation sites in the third cytoplasmic loop (Liggett et al, 1992), suggesting the possibility of homologous desensitization by ßARK for Gi/Go-coupled receptors. This speculation is supported by the fact that ßARK also induced phosphorylation of α2-adrenergic receptor (Liggett et al, 1992).

PART IV The Subtypes of the 5-HT Receptors and Their Signal Transduction Pathways

I. 5-HT Receptor Subtypes and Their Signals

Knowledge of the presence of multiple 5-hydroxytryptamine (5-HT, serotonin) receptors came first from experiments on the isolated guinea pig ileum (Gaddum & Picarelli, 1957). In the 1970s, development of radioligand binding methods furthered our understanding of the subtypes of receptors for 5-HT. Greater understanding has been given by molecular cloning and characterization of multiple genes encoding variety of 5-HT receptor subtypes. According to their pharmacological profiles and signalling phenotypes, 5-HT receptors have been divided into four major classes (Table 1), designated as 5-HT1-like, 5-HT2 (Julius et al, 1990), 5-HT3 (Bradley et al, 1986), and 5-HT4 (Dumuis et al, 1988).

The term 5-HT1-like, with high affinity binding to 5-HT (Ki $<$ 100 nM), was proposed for a heterogeneous group of receptors whose activation by 5-HT elicits functional responses that are: 1) potently blocked by methiothepin and/or methysergide (two drugs that show high affinity to 5-HT1 receptors; 2) not antagonized by ketanserin, a selective 5-HT2 antagonist, and MDL 72222, ICS 205-930, the selective 5-HT3 antagonist; and mimicked by 5-CT, a selective 5-HT1 agonist (Table 1).

Following successful molecular cloning of ß-adrenergic receptor, the first 5-HT1-like receptor cDNA clone, encoding human 5-HT1A receptor was isolated using lower stringency conditions (Kobilka et al, 1987; Fargin et al, 1988). At present, seven cDNAs encoding different 5-HT1-like receptor subtypes have been isolated and characterized from human and rat cDNA library or genomic DNA library (Table 2), including 5-HT1A (Kobilka et al, 1987; Fargin et al, 1988, Albert et al, 1990), 5-HT1B and its variant (Maroteaux et al, 1992, Jin et

al, 1992); 5-HT1C (Julius et al, 1988), 5-HT1Dα and 5-HT1Dß (Hamblin & Metcalf,1991; Demchyshyn et al, 1992), and 5-HT1E (McAllister et al, 1992). These six 5-HT1-like receptors are encoded by the different genes and differ in pharmacological properties, tissue distribution and transmembrane signalling systems, as well as biological functions (Julius 1991; Maroteaux et al, 1992;).

Comparison of the deduced amino acid sequences of the cloned 5-HT1-like receptor show a considerable amount of similarity. Within the transmembrane domains, the overall identity between 5-HT1A, 5-HT1B, 5-HT1D, and 5-HT1E receptors is about 50% (Demchyshyn et al, 1992; McAllister et al, 1992). This may explain why these receptors also share similar pharmacological profiles, eg. high affinity binding to 5-HT and 5-CT. By contrast, the overall homology in transmembrane domains of the 5-HT1C receptor with the other 5-HT1-like receptors is only 35% (Julius, 1991); unlike the other 5-HT1-like receptors, the deduced amino acid sequence of the 5-HT1C receptor share the great similarity (about 75% within transmembrane domains) with 5-HT2 receptor (Julius, 1991).

II. Cellular Signalling of 5-HT Receptors

5-HT was reported to couple to stimulate and inhibit cAMP synthesis in various tissue preparations, including brain slices and membrane preparations from variety of brain regions, eg. hippocampus, substantia nigra, and cortex (Barbaccia et al 1983; Shenker et al, 1985; De Vivo, Maayani, 1986; De Vivo & Maayani, 1988). Subtypes of 5-HT receptor which couple to inhibition of adenylyl cyclase are 5-HT1A (Shenker et al, 1987;), 5-HT1B (Bouhelal et al, 1988), and 5-HT1D (Schoeffter et al 1989), and 5-HT1E (Leonhardt et al, 1989). Stimulation of the 5-HT1B receptor endogenously expressed in fibroblast cells from Chinese hamster lung,

opossum kidney (OK) cells and primary culture cortical neurons induced inhibition of adenylyl cyclase activity stimulated by forskolin or parathyroid hormone, and PTX pretreatment blocked these actions (Munphy et al, 1988a; 1988b; Xu & Cheung, 1987). In rat brain, 5-HT1B receptor-mediated inhibition of forskolin-stimulated cAMP synthesis is correlated with the presence of 5-HT1B binding sites in the serotonergic terminal field, suggesting the possible role of 5-HT1B receptor-mediated inhibitory cAMP signal in neurotransmitter release. In calf substantia nigra, a good correlation was reported between the 5-HT1D receptor-induced inhibition of adenylyl cyclase activity and the potency of the agonist to inhibit evoked [3H]-5-HT release (Hoyer et al 1989).

Further evidence of inhibition of cAMP synthesis by these 5-HT1-like receptor was obtained by transfection of expression vectors for each cloned 5-HT1-like receptor into mammalian cell lines; it was showed that 5-HT1A, 5-HT1B, 5-HT1D and 5-HT1E receptors all induced PTX-sensitive inhibition of forskolin-stimulated adenylyl cyclase activity (Fargin et al, 1990; Raymond et al, 1989; Albert et al, 1990; Demchyshyn et al 1992).

Initially, the 5-HT_2 receptor was shown to link to stimulation of adenylyl cyclase activity in rabbit cerebellum (Kakiuchi & Rall, 1976), and subsequently, it was reported that the receptor positively links to adenylyl cyclase activity in several other brain regions, including hippocampus, cortex (Nelson et al, 1988). The subtype of 5-HT receptor which couples to stimulation of adenylyl cyclase remains to be determined. Based on the receptor binding studies in hippocampus, 5-HT1A receptor was positively identified to stimulate basal adenylyl cyclase activity (Shenker et al, 1987); however, the selective 5-HT1A receptor agonist, 8-OH-DPAT, rather inhibited cAMP synthesis in hippocampus. Unlike the Gs-mediated activation of adenylyl

cyclase, the stimulatory action of the 5-HT1A receptor on adenylyl cyclase was very weak, basal adenylyl cyclase activity increased by two- to three-fold (Dumuis et al, 1988a). Recently, the 5-HT7 receptor has been identified, and shown to stimulate adenylyl cyclase (Plassat et al, 1993; Lovenberg, T.W. et al, 1993). The presence of this Gs-coupled 5-HT receptor remains to be identified. It is also possible that 5-HT1A receptor-mediated stimulation by adenylyl cyclase in hippocampal neurons is tissue-specific signal due to the differences of cellular signalling milieu between hippocampal neuron and in the other brain regions where the 5-HT1A receptors are located.

5-HT1B and 5-HT1D receptors have similar pharmacological profiles (Frazer et al 1990) except for cynopindadol. Both receptors have been shown to be autoreceptors to inhibit 5-HT release in several brain regions (Hoyer et al 1989; Frazer et al 1990). In rat locus coeruleus, activation of 5-HT1B inhibits glutamate and GABA-mediated synaptic potentials (Engel, et al 1986). In guinea pig propositus hypoglossi, activation of presynaptic 5-HT1D receptor reversed the 5-HT-mediated inhibitory postsynaptic potentials. Thus 5-HT1B/5-HT1D receptor can inhibit the release of several neurotransmitter release including 5-HT.

Unlike the other 5-HT1-like receptors, the 5-HT1C receptor does not inhibit cAMP synthesis, but couples to stimulation of phospholipase C causing opening of chloride channels (Julius 1989). Injection of 5-HT1C receptor mRNA from choroid plexus tumor cells into frog oocytes initiated a serotonin-stimulated chloride current (Julius et al, 1988).

5-HT_2 receptor was the first 5-HT receptor shown to link to an increase PI turnover in guinea pig ileum (Jaffarji et al, 1986). The availability of selective drugs enabled a positive identification of the 5-HT-mediated stimulation of PI hydrolysis to 5-HT_2 receptor in muscle and

smooth muscle cells, platelets, astrocytes, C6 glioma cells (Coty et al, 1987; 1988; 1989; Hansson et al 1985; Ananth et al, 1987; Conn et al, 1985). In WRK1 cells, 5-HT induced a dose-dependent increase in PI turnover either in intact cell or in membrane preparations (Cory et al 1989). The action of 5-HT on PI turnover was blocked by ketanserin, a selective 5-HT_2 antagonist, and required the presence of GTP in the membrane preparation, suggesting mediation by a G protein (Coty et al, 1989). Further evidence for stimulation of PI turnover by 5-HT_2 receptor was from transfection of the cloned 5-HT_2 receptor gene in embryonic kidney 293 cells. Stimulation the transfected 5-HT_2 receptor induced an increase in PI hydrolysis and [Ca++]i.

In the CNS, activation of the 5-HT_2 receptor induced neuronal excitation (Bobker & Williams, 1990). In the rat, iontophoretical application of 5-HT to facial motoneurons caused a slow-onset depolarization and an increase in input resistance. 5-HT_2 receptor agonists, often hallucinogenic, also induced depolarization which was antagonized by ketanserin, a selective 5-HT_2 receptor antagonist. The depolarization induced by 5-HT_2 receptors activation was thought to be the result of decrease of potassium conductance. In rat nucleus accumbens neurons, activation of 5-HT_2 receptor induced an inward current at the resting membrane potential that reversed polarity of the potassium equilibrium potential (North & Ucimura, 1989). Nucleus accumbens neurons have a high resting membrane potential due in part to the activated state of the inward-rectifier potassium channels.

5-HT caused a rapid (<30ms), short-duration (100-300 ms) depolarizing response in several brains regions, which appeared to be due to direct gating ionic channels (Julius et al 1992). The 5-HT receptor subtype which mediated this response is known as the 5-HT_3 receptor. 5-HT_3 receptors are characterized by low affinity for 5-HT, and high affinity binding

to ICS 205-930, a selective 5-HT_3 antagonist; and are present in both CNS and peripheral neurons. 5-HT applied iontophoretically to neurons of the rabbit nodose ganglia caused a rapid depolarization, under voltage clamp, activation of 5-HT_3 receptor induced an inward current at the resting membrane potential and an increase in conductance, which has the properties of a cationic, non-selective, ligand-gated channel (Maricq et al, 1992). The 5-HT_3 receptor-activated depolarizing current has several characteristics in common with that activated by the nicotinic acetylcholine receptor. First, activation results in the opening of a cation channel. Second, sustained application of agonist produces a rapid desensitization. Finally, the conductance can be blocked by curare. These similarities suggest that 5-HT_3 receptor, like nicotinic receptor is a receptor-gated ion channel. A 5-HT_3 receptor cDNA has been isolated by screening a neuroblastoma expression library for functional expression of serotonin-gated currents in Xenopus oocytes (Maricq et al, 1992). The predicted protein shares many features with other members of the ligand-gated ion channel family. mRNA encoding the 5-HT3 receptors is found in the brain, spinal cord, and heart (Maricq et al, 1992).

The presence of the 5-HT_4 receptors was suggested by the finding that BRL 24924, a potent 5-HT_3 receptor antagonist induced activation of adenylyl cyclase in mouse embyro colliculi neurons in primary culture (Dumuis et al 1989). The 5-HT_4 receptor has low affinity for 5-HT_3 receptor agonists and antagonists such as ICS 205 930, MDL 72222, and were assumed to be involved in gastric and ileum motility which is specifically stimulated by benzamide derivatives (Dumuis et al 1989).

III. Cell Physiology of the 5-HT1A Receptor

Pharmacologically, the 5-HT1A receptor is classified by its high affinity binding of 5-HT

and 5-CT, and selective binding and activation by 8-OH-DPAT (Frazer et al, 1990). Unfortunately, in physiological studies, 8-OH-DPAT can act as a full agonist, a partial agonist, or even an antagonist depending on the receptor reserve of tissue. Nevertheless, when used at concentrations under 1 uM in combination with antagonist spiperone, 8-OH-DPAT identifies 5-HT1A-mediated responses (Frazer et al 1990).

The distribution of 5-HT1A receptors has been examined by receptor autoradiographic technique using [3H]8-OH-DPAT (Pazos & Palacios, 1985), by immunoautoradiography using an antibody against a synthetic peptide derived from the predicted sequence of the rat 5-HT1A receptor (El Mestikawy et al, 1990); and by in situ hybridization histochemistry using the synthetic oligonucleotides derived from the coding region of the rat 5-HT1A receptor gene as the probes (Pompeiano et al, 1991). 5-HT1A receptors are expressed only in neuronal tissue, mainly expressed in the dorsal raphe nucleus, septum, hippocampus, entorhinal cortex, and interpeduncular nucleus (El Mestikawy et al, 1990; Pompeiano et al, 1991). It is also detectable in the other brain regions; such as the olfactory bulb, cerebral cortex, some thalamic and hypothalamic nuclei, several nuclei in the brainstem, including all remaining raphe nuclei , nucleus of the solitary tract, and nucleus of the spinal tract of the trigeminus, and the dorsal horn of the spinal cord (El Mestikawy et al, 1990; Pompeiano et al, 1991).

A presynaptic localization of 5-HT1A receptors on the cell body dendrites of serotoninergic neurons in the dorsal raphe is supported by biochemical, lesion, and electrophysiological studies (Hall et al, 1985) and was further confirmed by in situ hybridization (Pompeiano et al 1991). In the septum, 5-HT1A receptor is expressed at the high levels in both cholinergic and GABAergic neurons. In hippocampus, the strong signal of the 5-HT1A receptor

mRNA was found in the granule cells and the pyramidal cells at CA1 and CA4 and in the most ventral levels of CA2 and CA3 (Pompeiano et al, 1991).

The 5-HT1A receptor has been shown to link to both activation (Shenker et al, 1985; Markstein et al, 1986) and inhibition of adenylyl cyclase (De Vivo & Maayani, 1986; Dumuis et al, 1988; Schoeffter & Hoyer, 1988); opening of potassium channels (Sprouse & Aghajanian, 1988) and closure calcium channels (Penington & Kelly, 1992). Except for stimulation of cAMP synthesis, all other inhibitory signals induced by 5-HT1A receptors are mediated through PTX-sensitive G proteins.

Activation of 5-HT1A receptor has been shown to inhibit forskolin-stimulated adenylyl cyclase activity in several different brain regions, including hippocampus, raphe nucleus, cortical neurons (De Vivo et al, 1986; Harrington et al 1988). This 5-HT inhibitory action was mimicked by 8-OH-DPAT, and was blocked by 5-HT1A receptor antagonist, such as spiperone (De Vivo et al, 1986); and was sensitive to PTX (Innis & Aghajanian, 1987; Innis et al 1988), suggesting mediation by Gi/Go.

In hippocampal CA1 neurons, activation of 5-HT1A receptor caused a hyperpolarization, which was a result of increase in potassium channel conductance (North et al, 1987; Gahwiler & Brown, 1985; Bobker & Williams, 1990). In isolated hippocampal pyramidal cells, it was found that 5-HT1A receptor activation evoked a GTP-sensitive potassium current, which was blocked by PTX-pretreatment (North et al, 1987; Andrade et al, 1986; Andrade & Nicoll 1987). The release of 5-HT from the terminals of serotonergic raphe neurons is under inhibitory feedback control. 5-HT, acting on raphe cell body 5-HT1A autoreceptors, mediates inhibitory postsynaptic potentials as a result of release of 5-HT from collaterals from neighbouring raphe

neurons. In rat dorsal raphe, activation of the 5-HT1A receptor induced an inhibitory postsynaptic potential (IPSP), this action was blocked by spiperone, prolonged by cocaine and fluoxetine, and reversed polarity at the potassium equilibrium potential (Clarke et al, 1987). Using voltage-clamp technology, 5-HT has been shown to differentially induce three types of potassium currents: a calcium-independent potassium current which was not responsible for neuronal hyperpolarization and it was an inhibitory signal; a calcium-dependent potassium current which was largely responsible for cell firing in CA1 neurons and produced a paradoxical increase in neuronal discharge in response to a depolarizing input; and a slowly developing and long-lasting suppression of an intrinsic voltage-dependent-potassium current, leading to neuronal depolarization and excitation (Colino & Halliwell, 1987). Pharmacological analysis using selective agonists and antagonists for different subtypes of 5-HT receptor indicate that the calcium-dependent potassium current appeared to be mediated by 5-HT1A receptor; the 5-HT receptors which mediate the other two potassium currents remain to be identified (Colino & Halliwell, 1987).

Opening of potassium channels by 5-HT1A receptor activation in raphe neurons may indirectly reduce calcium influx due to the decrease in action potential frequency. Using acutely isolated, patch-clamped dorsal raphe neurons, activation of 5-HT1A receptor induced inhibition of calcium currents (Penington & Kelly, 1991). Application of 5-HT reduced the size of calcium currents elicited at -100 or -50 mV; the action of 5-HT was mimicked by 8-OH-DPAT, and was blocked by NAN 190, a 5-HT1A receptor antagonist (Penington & Kelly, 1991). Decrease of calcium influx during a raphe neuron action potential will in turn reduce a component of the spike after hyperpolarization thought to be a calcium-activated potassium conductance in

hippocampal neurons. Any change of this current in raphe neurons will alter the amount of 5-HT release (Penington & Kelly, 1991). One interesting phenomenon is that the hyperpolarization mediated by 5-HT1A receptor in raphe neurons is conducted by both increase of potassium currents and decrease of calcium currents, while in hippocampal neurons, the hyperpolarization was mediated solely by opening of potassium channels (Colino & Halliwell, 1987; Penington & Kelly, 1991).

The biochemical mechanisms of 5-HT1A receptor transmembrane signalling were further investigated by transfection of the cloned human 5-HT1A receptor gene in COS-7 or HeLa cells both of which lack the receptor. Stimulation of 5-HT1A receptors in both cell systems activated multiple signal transduction pathways (Fargin et al, 1989; Raymond et al, 1989; Raymond et al, 1991). In Hela cells, activation of the expressed human 5-HT1A receptor markedly inhibited both the ß2-adrenergic receptor- or forskolin-stimulated adenylyl cyclase, activated phospholipase C, increased protein kinase C activity and stimulated sodium-dependent phosphate uptake (Raymond et al, 1989; Raymond et al, 1991). In COS-7 cells, stimulation of the expressed 5-HT1A receptor decreased adenylyl cyclase activity and increased the phospholipase C activity (Fargin et al, 1989), both actions were mediated by PTX-sensitive G proteins (Fargin et al, 1989). These results indicate that 5-HT1A receptor can mediated multiple signal transduction pathways in a given cell system, and it may induce distinct signals in different cell systems.

The specificity of 5-HT1A receptor coupling to different G protein was investigated using an in vitro reconstitution system based on the expression of recombinant 5-HT1A receptor and G protein α and ßγ subunits in Escherichia coli (Bertin et al, 1992). This study showed that incubation of Gα.ßγ with E. coli membranes expressing the rat 5-HT1A receptor resulted in

high affinity agonist [3H]8-OH-DPAT binding; and titration with a panel of Gα subtypes showed the order of potency: αi3 > αi2 > αi1 > αo; while αs failed to interaction with 5-HT1A receptor (Bertin et al, 1992).

Table 3. The nomenclature of serotonin receptor classification

	Agonist	Antagonist	Cellular signals
5-HT-like	5-CT	Methysergide	
	5-HT	Methiothepin	
5-HT1A	8-OH-DPAT		cAMP↓, K+↑, Ca++↓
5-HT1B	Cyanopindolol		cAMP↓, K+↓?
5-HT1C		Methysergide	PI ↑, Cl^-↑
5-HT1D	5-CT		cAMP↓
5-HT1E	5-HT (low affinity to 5-CT)		cAMP↓
5-HT2		Ketanserin Methysergide	PI↑, Cl↑, K+↓
5-HT3	2-Methyl-5-hydroxytryptamine	MDL 72222 ICS 205-930	ligand-gated ion channel
5-HT4	BRL 24924	ICS 205-930	cAMP↑

Abbreviation: 5-CT, 5-carboxamidotryptamine; 5-HT, serotonin; 8-OH-DPAT, 8-hydoxy-(2-(N,N-dipropylamino)-tetraline.

PART V The Subtypes of Dopamine Receptors and Their Signal transduction Pathways

The early studies suggested the existence of at least two different subtypes of dopamine receptors, termed as D1 and D2 (Kebabian & Greengard, 1971; Kebabian et al, 1972; Seeman et al, 1975; Seeman, 1980). Dopamine-D1 receptor acts as a stimulatory receptor and induces stimulation of adenylyl cyclase (Kebabian & Greengard, 1971; while dopamine-D2 receptor acts as an inhibitory receptor and mediates inhibition of adenylyl cyclase, opening of potassium channels and closure of calcium channels (Seeman, 1980; Sibley & Monsma 1991; Civelli et al, 1993).

The use of recombinant DNA techniques has resulted in the identification and the cloning of multiple dopamine receptors (Table I). Five pharmacologically different dopamine receptors have been now cloned. The dopamine receptor family is even larger due to the presence of alternative splicing RNAs of some subtypes of dopamine receptors (Civelli et al 1993).

I. Dopamine-D2 Receptor

Using the full-length of ß-adrenergic receptor cDNA as a probe to screen a rat genomic DNA library at the low-stringent hybridization conditions, the first dopamine receptor, the rat dopamine-D2S receptor was cloned (Bunzow et al, 1988). The cloned dopamine-D2 receptor cDNA encodes a 415 amino acid protein with all the typical characteristics of G protein coupled receptors: seven hydrophobic domains, 21 amino acid residues which are conserved among all G protein coupled receptors and a significant sequence similarity with the other G protein coupled receptors, and potential glycosylation sites (Bunzow et al, 1988). When expressed in Ltk- fibroblast cells, the cloned receptor exhibited appropriate radioligand binding activity with pharmacological characteristics of the D2 receptor. The human D2 receptor was subsequently

cloned and shown 96% identical to the rat receptor, with one amino acid deletion (Grandy et al, 1990).

Shortly after the cloning of the first dopamine D2 receptor, it was discovered that this receptor exists in two isoforms that differ in length by 29 amino acids at the third cytoplasmic loop and are derived from the same gene by alternative RNA splicing (Giros et al 1989; Monsmam et al, 1989; Grandy et al, 1990).

The tissue distribution of dopamine-D2 receptors has been determined by autoradiography Northern blot, in situ hybridization, immunochemistry using the antibodies against different peptides derived from C-terminus and the third cytoplasmic loop of the receptor (Bunzow et al, 1988; Weiner & Brann 1989; Gerfen et al 1990; McVittie et al, 1991). Expression of dopamine D2 receptors is restricted to neurons and endocrine cells (McVittie et al, 1991). The highest expression in rat brain was found in striatal and mesolimbic regions, corresponding to the major dopaminergic projection areas including caudate putamen, nucleus accumbens and olfactory tubercle (Gerfen et al, 1990). The long form of the D2 receptor is predominantly expressed in all regions where the D2 receptors are detected, although the exact ratio of the two D2 receptor subtypes can vary significantly (McVittie et al 1991).

In the striatum, the D2 receptors are localized on medium-size neurons where they are co-expressed with enkephalin. The D2 receptor mRNA was also found in the large-diameter neurons, which are mainly cholinergic interneurons (Gerfen et al 1990; McVittie et al, 1991).

It is well-established that native dopamine D2 receptors induce inhibition of adenylyl cyclase in membrane preparations, and in intact cells (Civelli et al, 1993). Expression of the cloned dopamine receptor cDNA in variety of cell lines further demonstrated that the D2

receptor inhibited unstimulated and Gs-coupled receptor- or forskolin-stimulated adenylyl cyclase activity in both membrane preparation and in intact cells (Albert et al 1990; Vallar et al 1991). Inhibition of adenylyl cyclase induced by the D2 receptor was sensitive to PTX pretreatment, indicating that the action is mediated by Gi/Go (Vallar et al 1991). In cultured pituitary lactotrophs and GH4C1 cells, the D2 receptor-induced inhibition of adenylyl cyclase is well correlated with its inhibitory action on prolactin secretion induced by TRH, BAY K8644 or VIP (Enjalbert et al, 1988). It was initially believed that the dopamine D2 receptor-induced inhibition of prolactin secretion in pituitary is mediated solely by its action on adenylyl cyclase. However, recent studies have indicated diversity of signal transduction pathways mediated by D2 receptors; and each of these pathways may play an important role in the receptor-induced biological as well as physiological actions.

Like the other Gi/Go coupled receptors, the dopamine D2 receptor also induced activation of potassium channels. In lactotrophs, dopamine induced a PTX-sensitive hyperpolarization in a concentration-dependent manner, with an EC50 similar to that obtained for inhibition of adenylyl cyclase activity (Malgaroli et al 1987; Israel et al, 1987). In GH4C1 cells which lack native D2 receptors, activation of the transfected D2S receptors also induced hyperpolarization (Vallar et al 1990). Further studies using patch-clamp recording of the whole cell demonstrated that dopamine induced two ligand-activated potassium currents in rat pituitary lactotroph and melanotroph cells (Lldeo et al, 1991). The action of dopamine required the presence of intracellular GTP, and was potentiated by GTPγS, blocked by PTX-pretreatment (Lldeo et al, 1990a). In striatal slices, dopamine D2 receptor inhibited high-K+-induced increase in [Ca++]i (Lldeo et al 1990b). In snail neurons and in *Aplysia* abdominal ganglia, activation of

dopamine receptors increased potassium conductance and hyperpolarization (Vallar & Meldolesi, 1990).

It is well-established that dopamine-D2 receptors inhibited calcium conductance in neurons and in endocrine cells (Lldeo et al 1990a; 1990b). In sensory neurons, dopamine was reported to block T-type calcium channels and to delay L-type calcium channels (Lldeo et al 1990b). In the small-size neurons, activation of dopamine D2 receptor has been shown to inhibit calcium currents. In lactotroph cells, dopamine concomitantly reduced two different voltage-dependent calcium currents in addition to increasing potassium currents (Lledo et al, 1990a; 1990b). The action of D2 receptors on calcium channels was potentiated by GTPγS, and was abolished by PTX-pretreatment, suggesting that this action was mediated by Gi/Go.

In pituitary lactotroph cells, the D2 receptors are negatively coupled to phospholipase C activity. Pretreatment of lactotroph cells with dopamine inhibited TRH or angiotensin II receptor-induced stimulation of phospholipase C activity (Malgaroli et al, 1987; Vallar & Meldolesi, 1990). Dopamine only inhibited the plateau but not the peak of increase of PI turnover induced by TRH or angiotensin, suggesting that the D2 receptor may not directly inhibit PI turnover. Using the purified pituitary lactotroph cells loaded with fura-2, dopamine was shown to inhibit the TRH-induced transient increase in [Ca++]i in calcium-free buffer (Malgaroli et al, 1987). The action of dopamine can be reversed by any treatment that increases intracellular cAMP levels, such as forskolin, VIP, CTX etc., suggesting that the D2 receptor-mediated inhibition of phospholipase C is secondary to its inhibitory action on adenylyl cyclase (Malgaroli et al, 1987; Vallar & Meldolesi, 1990).

The multiplicity of signal transduction pathways of the D2 receptors was further

investigated by expression of the cloned D2 receptor cDNA in GH4C1 and Ltk- cells. In GH4C1 cells, activation of transfected D2S receptor induced inhibition of cAMP accumulation, calcium influx, and high-K-induced hyperpolarization, the actions of dopamine were sensitive to PTX (Vallar et al 1990; Albert et al, 1990). While in Ltk- fibroblast cells, the D2 receptor activation induced a novel activation of phospholipase C activity in addition to inhibition of adenylyl cyclase (Vallar, et al, 1990). The action of dopamine on PLC was also sensitive to PTX-pretreatment, suggesting the mediation by Gi/Go. Moreover, stimulation of the D2 receptors expressed in CHO cells had no effect on phospholipase A2 activity, but significantly potentiated activation of this enzyme induced by thrombin, ATP etc. (Kanterman et al, 1990).

Given the fact that there are four different PTX-sensitive G proteins, Go, Gi1, Gi2, Gi3; and that Go exists in two isoforms Go1 and Go2 due to the alternative splicing of RNA (Strathmann et al, 1990; Tsukamoto et al 1991), the specificity of Gi/Go in coupling of the D2 receptor and various effectors remains unclear at present. Studies using reconstitution approach shown that the purified pituitary D2 receptor can fully activate Gi2, partially activated Gi1 and Gi3, but had no effect on Go (Senogles et al, 1990). However, patch-clamp recordings in the whole-cell configuration with injection of different antibodies against $\alpha o/\alpha i$ subunits demonstrated that αo antiserum inhibited the D2 receptor-mediated calcium currents, while $\alpha i3$ antiserum suppressed the receptor-induced potassium currents. On the other hand, injection of $\alpha i2$ antiserum had no effect on the D2 receptor-induced calcium and potassium currents (LIedo et al, 1990a). Thus, the specificity of the D2 receptor coupling to G proteins and the difference between the two forms of the D2 receptor in interaction with G proteins remain to be determined

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II. Dopamine-D1 Receptor

Following the cloning of the D2 receptors, the human and rat dopamine-D1 receptor cDNAs were simultaneously cloned by three different groups (Dearry et al, 1990; Sunahara et al, 1990; Zhou et al 1990). Like the D2 receptors, the D1 receptor is also a member of seven-transmembrane-domain receptor family and induces cellular signals via G proteins. The human and rat D1 receptors are both 446 residues in length; and they have overall 91% identity. The gene of human receptor is localized at chromosome 5, and is intronless (Sunahara et al 1990). The mRNA of the D1 receptor is mainly detected in caudate putamen, nucleus accumbens, and olfactory tubercles (Dearry et al 1990; Zhou et al, 1990).

It is well established that the dopamine D1 receptors induces activation of adenylyl cyclase. Activation of the D1 receptors has been shown to stimulate adenylyl cyclase activity in membranes from rat striatum and kidney proximal tubules (Stoof & Kebabian, 1981; Felder et al, 1988), and in several intact cell systems including rat retinal horizontal neurons (Rodrigues & Dowling, 1990), COS-1 cells (Steffey et al, 1992); human astrocytoma (Balmforth et al, 1988), and a mouse neuroblastoma cell line (Monsma et al, 1989). Expression of the clone D1 receptor cDNA in COS-7 and in Ltk- cells has verified that this receptor can effectively stimulate adenylyl cyclase (Dearry, et al, 1990; Zhou et al, 1990).

However, numerous studies have revealed that the D1 receptor may induce other signals in addition to elevating cAMP levels. In renal tubular membrane preparations, the D1 receptor agonist, SKF 82526, but not the D2 receptor agonist, was shown to stimulate PLC activity (Felder et al, 1988, 1989). The action of the D1 receptor on PLC was blocked by the selective D1 receptor antagonist SCH 23390, but not by the other receptor antagonists, and can not be

mimicked by forskolin or non-hydrolysable cAMP analogues and was enhanced by GTP analog. These results indicate that the D1 receptor may independently regulate both PLC and adenylyl cyclase activity via G proteins (Felder et al, 1988,1989). In retinal horizontal cells, the D1 receptors were shown to induce cAMP-dependent neurite retraction via DAG and protein kinase C (Rodrigues & Dowling, 1990). Moreover, the solubilized D1 receptor from rat striatum coupled to both CTX and PTX sensitive G proteins in reconstituted phospholipid vesicles (Sidhu et al, 1991).

III. Dopamine-D3 Receptor

Using probes derived from the rat D2 receptor sequences, the D3 receptor was cloned from rat cDNA library and the gene encodes a protein of 446 amino acids with 52% homology to the D2 receptor (Sokoloff et al, 1990). The gene encoding the human D3 receptor has been also characterized; and surprisingly, the human receptor has 46 fewer amino acids in the third cytoplasmic loop. The human D3 receptor has overall 88% amino acid homology to the rat receptor, and within the transmembrane domains, the homology is 97% (Sibley et al, 1991).

Pharmacologically, the D3 receptor resembles, but is distinct from the D2 receptor. The transfected rat D3 receptor in CHO cells exhibited higher affinity for dopamine (20-fold) and quinpirole (100-fold) than the D2 receptor; on the other hand, the D2 receptor shows greater affinity to most of the D2 receptor antagonists (Sokoloff et al 1990). Interestingly, the agonist binding of the D3 receptor in CHO cells is insensitive to GTP; moreover, activation of the D3 receptor had no effect on adenylyl cyclase activity in CHO cells (Giros et al, 1992). It is possible that CHO cell lacks appropriate G proteins or effectors which couple to the D3

receptor.

The D3 receptor is mainly expressed in limbic brain areas, including olfactory tubercles, nucleus accumbens, islands of Calleja and hypothalamus. The pattern of the D3 receptor localization indicated the this receptor may be important for dopaminergic control of cognitive and emotional functions.

IV. Dopamine-D4 Receptors

Using the rat D2 receptor cDNA as the probe to screen a human neuroblastoma cell cDNA library at the low-stringency , the D4 receptor cDNA encoding a protein of 387 amino acids was cloned and sequenced (Van Tol et al 1991). Structurally, the D4 receptor resembles both the D2 and the D3 receptors, with seven putative transmembrane domains and a relatively short third cytoplasmic loop. The overall homology of the D4 receptor to the D2 and D3 receptors is 41% and 39%, respectively; within the membrane-spanning domains, the homology to both receptor is 56%. Like the D2 or D3 receptor, the D4 receptor contains one consensus cAMP-dependent phosphorylation site in the third cytoplasmic loop, and a conserved cysteine residue in the C-terminus (Van Tol et al, 1991; Civelli et al 1993).

In comparison with the binding activity of the D2 receptor, the transfected D4 receptor in COS-7 cells exhibited similar binding activity to most of dopamine receptor agonists or antagonists. Interestingly, clozapine, an atypical antipsychotic drug and its congener, clorotepine, displayed about 10-fold higher affinity for the D4 receptors, suggesting the importance of this receptor in schizophrenia. The binding of the D4 receptor to dopamine is sensitive to GTP (Van Tol et al, 1991). The signal transduction pathways for the D4 receptor remain to be determined.

The mRNA for the D4 receptor is mainly detected in the frontal cortex, midbrain, amygdala and medulla, with low levels observed in the striatum and olfactory tubercles. The tissue distribution of the D4 receptor explains the lack of extrapyramidal side effects observed with clozapine treatment.

V. Dopamine-D5 Receptor

Using a fragment from the D1 receptor cDNA as the probe, the fifth dopamine receptor, the dopamine D5 receptor, was cloned from human genomic library (Sunahara et al, 1991). The gene encodes a protein of 447 amino acids. Like the other G protein coupled receptor, the D5 receptor also contains seven putative tranmembrane-spanning domains, a cAMP-dependent potential phosphorylation site in the third cytoplasmic loop, and a conserved cysteine in the C-terminus (Sunahara et al 1991). The overall homology of the D5 receptor to the D1 receptor is about 50%; within the transmembrane-spanning regions, the homology is over 80% (Civelli et al 1993).

The binding activity of the D5 receptor in COS-7 cells resembles that of the D1 receptor except that the D5 receptor exhibited about ten-fold higher affinity to dopamine than the D1 receptor. The interaction of the D5 receptor with dopamine is not sensitive to GTP in COS-7 cells, but the receptor is coupled to stimulation of adenylyl cyclase when expressed in GH4C1 cells (Sunahara et al 1991).

The highest expression areas of the D5 receptor mRNA are hippocampus, hypothalamus, as well as mamilliary and pretectal nuclei, little mRNA is found in the striatum.

Table 4. The subtypes of dopamine receptors.

The Nomenclature	Human chromosome localization	Signalling pathways	Tissue Distribution
D1	5	cAMP↑	CP, NA, OT
D2S/D2L	11	cAMP↓ Ca++↓ K+↑	CP, NA, OT
D3	3	cAMP↓?	OT, HPT, NA
D4	11	cAMP↓?	FC, medulla midbrain
D5	4	cAMP↑	HPC, HPT

Abbreviations: CP., caudate-putamen; NA., nucleus accumbens; OT, olfactory tubercle; HPT, hypothalamus; FC, frontal cortex; HPC, hippocampus.

CHAPTER III CELL-SPECIFIC SIGNALING OF THE 5-HT1A RECEPTORS

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Cell-specific Signaling of the 5-HT1A Receptor

MODULATION BY PROTEIN KINASES C AND A*

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Heterologous expression of the rat 5-HT1A receptor in stably transfected GH4C1 rat pituitary cells (clone GH4ZD10) and mouse Ltk⁻ fibroblast cells (clone LZD-7) (Albert, P. R., Zhou, Q.-Y., VanTol, H. H. M., Bunzow, J. R., and Civelli, O. (1990) *J. Biol. Chem.* 265, 5825–5832) was used to characterize the cellular specificity of signal transduction by the 5-HT1A receptor. We demonstrate that the 5-HT1A receptor, acting via pertussis toxin-sensitive G proteins, can change its inhibitory signaling phenotype and become a stimulatory receptor, depending on the cell type, differentiation state, or intracellular milieu of the cell in which it is expressed. When expressed in pituitary GH4ZD10 cells, activation of 5-HT1A receptors decreased both basal and vasoactive intestinal peptide-enhanced cAMP accumulation and blocked (±)-Bay K8644-induced influx of calcium, inhibitory responses which are typical of neurons which endogenously express this receptor. Similarly, 5-hydroxytryptamine (5-HT) also inhibited adenylyl cyclase in fibroblast LZD-7 cells, reducing the forskolin-induced enhancement of cAMP levels by 50%, but did not alter basal cAMP levels. In contrast to GH4ZD10 cells, where 5-HT had no effect on basal or thyrotropin-releasing hormone-induced phosphatidylinositol turnover, 5-HT enhanced the accumulation of inositol phosphates and induced a biphasic increase in $[Ca^{2+}]_i$ in LZD-7 cells. These dominant stimulatory actions of 5-HT, as well as the inhibitory effects, were absent in untransfected cells and displayed the potency and pharmacological specificity of the 5-HT1A receptor, indicating that the 5-HT1A subtype coupled to both inhibitory and stimulatory pathways in the fibroblast cell. The actions of 5-HT in GH and L cells were blocked by 24-h pretreatment with pertussis toxin, suggesting that inhibitory G proteins (G_i/G_o) mediate both inhibitory and stimulatory signal transduction of the 5-HT1A receptor. However, the 5-HT-induced stimulatory pathway in fibroblasts was blocked selectively by acute (2-min) pretreatment with TPA, an activator of protein kinase C. This action of protein kinase C was potentiated by activation of protein kinase A, indicating that the expression of the stimulatory pathway of the 5-HT1A receptor in LZD-7 cells is modulated by second messengers.

Many hormone and neurotransmitter receptors transduce and amplify their signal via coupling to heterotrimeric G proteins to change the activity of effector enzymes (*e.g.* adenylylcyclase or phospholipase C), which generate intracellular second messengers (Birnbaumer *et al.*, 1990; Gilman, 1987; Ross, 1989). Adenylylcyclase catalyzes the formation of cAMP, a second messenger which activates protein kinase A, inducing the phosphorylation of multiple substrate proteins. Phospholipase C-catalyzed hydrolysis of PIP_2[1] leads to formation of the second messengers IP_3 and DAG (Berridge and Irvine, 1989; Berridge, 1987; Colino and Halliwell, 1987). IP_3 elevates $[Ca^{2+}]_i$ by mobilizing nonmitochondrial calcium stores (Prentki *et al.*, 1984; Streb *et al.*, 1983). DAG induces activation of protein kinase C, and translocation of the enzyme from the cytosol to the membrane compartment, resulting in phosphorylation of a number of protein substrates (Nishizuka, 1986, 1988). PTx-sensitive G proteins are coupled to inhibition of these processes, although in certain cell types, PTx-sensitive enhancement of phospholipase C activity has been reported (Ross, 1989). Thus, there may exist a cellular specificity of the transduction pathways mediated by this class of heterotrimeric G proteins.

In cell types where receptor-induced enhancement of PI turnover was blocked by pretreatment with PTx, a parallel PTx-sensitive enhancement of cell proliferation was sometimes reported (Kavanaugh *et al.*, 1988; Seuwen *et al.*, 1988; Van Corven *et al.*, 1989), a response not observed in cells without this pathway (*e.g.* nondividing neuronal cells). Although it is sometimes unclear which intracellular messenger (*e.g.* cAMP, DAG, $[Ca^{2+}]_i$, or others) generates the proliferative signal (Van Corven *et al.*, 1989), these second messengers are known to play important roles in the proliferation and differentiation of cells (Berridge, 1987; Mendoza *et al.*, 1986; Weinstein, 1988). The capacity of a family of receptors to assume different signaling pathways depending on the cell type may play a role in determining the proliferative state of a cell or may participate in the differentiation process of dividing stem cells. At the very least, signal transduction switching would alter the response of a target cell to a given stimulus, possibly even reversing the response. Such altered responsiveness could influence an entire network of cells,

[1] The abbreviations used are: PIP_2, phosphatidylinositol (4,5)-bisphosphate; 5-HT, 5-hydroxytryptamine, serotonin; $[Ca^{2+}]_i$, cytosolic free calcium concentration; PI, phosphatidylinositol; IP_3, inositol (1,4,5)-trisphosphate; DAG, diacylglycerol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; TRH, thyrotropin-releasing hormone; VIP, vasoactive intestinal peptide; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; G protein, guanine nucleotide-binding regulatory protein; G_i, inhibitory G protein; G_o, G protein identified as a PTx substrate in brain; PTx, pertussis toxin; 8-Br-cAMP, 8-bromo-cyclic adenosine adenosine 3′:5 monophosphate; PLC, phospholipase C; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid.

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leading to subtle or perhaps great alterations in organism as a whole.

These studies were undertaken to determine whether a specific receptor, the 5-HT1A receptor, is coupled via PTx-sensitive G proteins to the same signal transduction pathway independent of the cell type in which it is expressed or whether there exists a cell specificity to the signal transduction pathway of the receptor. When expressed in GH4C1 pituitary cells, which have several properties of neuronal cells including pituitary hormone receptors, voltage-gated ion channels, and regulated secretion of hormones, the 5-HT1A receptor displayed a signaling phenotype characteristic of neurons in which the receptor is endogenously expressed. That is, 5-HT inhibited adenylylcyclase (Albert *et al.*, 1990; Clark *et al.*, 1987; Dumuis *et al.*, 1988; Fargin *et al.*, 1989; Zgombick *et al.*, 1989) and decreased calcium influx via voltage-gated calcium channels (Colino and Halliwell, 1987; Innis and Aghajanian, 1987; Pennington and Kelly, 1990; Ropert, 1988; Sprouse and Aghajanian, 1988; Zgombick *et al.*, 1989). When expressed in Ltk$^-$ fibroblast cells, a model of undifferentiated cells, the 5-HT1A receptor exhibited an additional stimulatory pathway, switching its signaling pathway from inhibition to stimulation of $[Ca^{2+}]_i$. Thus the 5-HT1A receptor displayed either the inhibitory or the dominant stimulatory signal transduction phenotype, depending on the cell in which it was expressed. The choice of signaling phenotype was modulated by activation of protein kinases C and A, converting the fibroblast signaling phenotype to neuron-like signaling.

EXPERIMENTAL PROCEDURES

Materials

TPA, 8-Br-cAMP, forskolin, and 5-HT, IBMX, and VIP were purchased from Sigma; TRH and ionomycin were from Calbiochem; *myo*-[2-^{3}H]inositol was obtained from Amersham Corp. Spiperone and (±)-Bay K8644 were from Research Biochemical Inc., Natick, MA. Fura 2-AM was from Molecular Probes, Eugene, OR. GH4C1 cells were from Dr. A. H. Tashjian, Harvard School of Public Health, Boston, MA. Ltk$^-$ cells were from Dr. O. Civelli, Oregon Health Sciences University, Portland, OR.

Methods

Cell Culture—All cells were grown as monolayer in α-minimum Eagle's medium supplemented with 5% fetal bovine serum (L cells) or Ham's F-10 medium with 8% fetal bovine serum (GH cells), at 37 °C in a humidified atmosphere, with 5.0% carbon dioxide. Media were changed 12–24 h prior to experimentation. The concentration of 5-HT present in the medium was at most 10 ± 4 nM (based on serum 5-HT concentrations provided by GIBCO). Chronic (24-h) treatment with 1 μM 5-HT did not alter the acute responses measured in 5-HT-free HBBS buffer (see below).

Measurement of $[Ca^{2+}]_i$—Cells were harvested by incubation in calcium-free HBBS containing 5 mM EDTA and 0.05% trypsin and centrifuged at 200 × *g* for 3 min at room temperature. The cells were washed once with HBBS (118 mM NaCl, 4.6 mM KCl, 1.0 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 20 mM Hepes, pH 7.2) and then resuspended in 0.5 ml of HBBS and incubated for 30 min at 37 °C in the presence of 2 μM Fura-2AM (dissolved in dimethyl sulfoxide to 5 mM). They were then diluted to 10 ml, centrifuged, washed twice with HBBS buffer, and finally resuspended in 2 ml of HBBS and placed in a fluorescence cuvette. Change in fluorescence ratio (λ_{ex} = 340/380 nm, λ_{em} = 500 nm) was recorded on a Perkin-Elmer (Buckinghamshire, Great Britain) LS-50 spectrofluorometer and analyzed by computer, based on a K_D of 227 nM for the Fura 2·Ca^{2+} complex (Grynkiewicz *et al.*, 1985). Calibration of R_{max} was performed by addition of 0.1% Triton X-100 and 20 mM Tris base and of R_{min} by addition of 10 mM EGTA (Albert and Tashjian, 1984; Grynkiewicz *et al.*, 1985). All experimental compounds were added directly to the cuvette from 200-fold concentrated test solutions at times indicated in the figures.

cAMP Assay—Measurement of cAMP was performed as described previously (Albert *et al.*, 1990). In brief, cells were plated in six-well 35-mm dishes; medium was changed 12–24 h prior to experimentation. After removal of the medium, cells were preincubated in 2 ml/well HBBS for 5–10 min at 37 °C, the buffer was replaced by 1 ml of HBBS containing 100 μM IBMX, and the incubation was continued for another 5 min. Then, the various test compounds were added to the wells, and the cells were incubated at room temperature for 30 min. The buffer was collected and stored at −20 °C until assay for cAMP. cAMP was assayed by a specific radioimmunoassay (ICN) as described (Albert *et al.*, 1990) with antibody used at 1:500 dilution. After 16 h of incubation at 4 °C, 20 μl of 10% bovine serum albumin and 1 ml of 95% ethanol were added consecutively to precipitate the antibody-antigen complex. Standard curves showed IC_{50} of 0.5 ± 0.2 pmol by using cAMP as standard. Data for cAMP assay are described as mean ± S.E. for triplicate wells.

Assessment of PI Hydrolysis—An adaptation of the protocols of Raymond *et al.* (1989) was used. In brief, cells grown in six-well dishes were equilibrated for 48 h in the regular medium supplemented with 3 μCi/ml of [^{3}H]*myo*-inositol. After washing three times with HBBS, cells were incubated for 2 h in HBBS containing 10 mM LiCl. The medium was then replaced by 1 ml of fresh medium supplemented by various concentrations of test compounds, and the cells were incubated for 25 min. The reaction was terminated by aspiration followed by the addition of 0.3 ml of ice-cold 10% trichloroacetic acid. The lysates were collected and centrifuged 10,000 × *g* for 15 min. The supernatants were extracted three times with 2.5 ml of diethyl ether, while the pellets were discarded. The samples were neutralized by addition of 10 μl of 1 N NaOH, and their radioactivity was measured. In our assay, basal IP levels were 480 ± 169 cpm/dish in GH cells and 29,000 ± 3000 cpm/dish (mean ± S.D. of five independent experiments).

Statistical Method—Data were analyzed for statistical significance using the Student's *t* test.

RESULTS

Inhibitory Signaling by 5-HT1A Receptors in Pituitary Cells—GH4ZD10 rat pituitary cells expressing the transfected rat 5-HT1A receptor (Albert *et al.*, 1990) were used to characterize the signal transduction pathways of the 5-HT1A receptor in an electrically excitable cell model. As shown previously (Albert *et al.*, 1990), treatment of these cells with 100 nM 5-HT significantly decreased both basal and VIP-stimulated cAMP accumulation; these actions of 5-HT were abolished by 24-h pretreatment with 25 ng/ml PTx (Fig. 1*A*). Concurrent addition of 100 nM TPA attenuated 5-HT-induced inhibition of basal cAMP accumulation but was without effect on inhibition of VIP-stimulated cAMP levels. Neither PTx nor TPA significantly decrease basal cAMP levels. Since accumulation of cAMP in our experiment was measured in the presence of 100 μM IBMX to inhibit cAMP degradation by phosphodiesterases (Albert *et al.*, 1990), the observed change in cAMP accumulation presumably represents a change in its synthesis. Thus, the 5-HT1A receptor induced a G_i/G_o-mediated inhibition of adenylate cyclase as observed in other cell types (Clark *et al.*, 1987; Dumuis *et al.*, 1988; Fargin *et al.*, 1989; Frazer *et al.*, 1990; Zgombick *et al.*, 1989).

In order to examine the actions of 5-HT1A receptor activation on voltage-dependent calcium influx, the opening of L-type calcium channels was enhanced using dihydropyridine channel agonist (±)-Bay K8644 (Enyeart *et al.*, 1987, 1990; Triggle and Janis, 1987), and changes in $[Ca^{2+}]_i$ were measured using intracellularly trapped fluorescent calcium indicator Fura-2 (Grynkiewicz *et al.*, 1985) (see "Methods"). (±)-Bay K8644 (200 nM) increased $[Ca^{2+}]_i$ by 1.4-fold in GH4ZD10 cells (Fig. 1*B*). Acute pretreatment with 1 μM 5-HT did not alter basal $[Ca^{2+}]_i$ but completely blocked the action of 200 nM (±)-Bay K8644 (Fig. 1*B*). This inhibitory action of 5-HT was abolished by 10 μM spiperone (not shown) or by 24-h pretreatment with 25 ng/ml PTx (Fig. 1*C*). In untransfected GH4C1 cells no effect of 5-HT (1 μM) on (±)-Bay K8644 actions was observed (not shown). Thus, as observed in neuronal systems, 5-HT1A receptor activation in GH4ZD10 cells

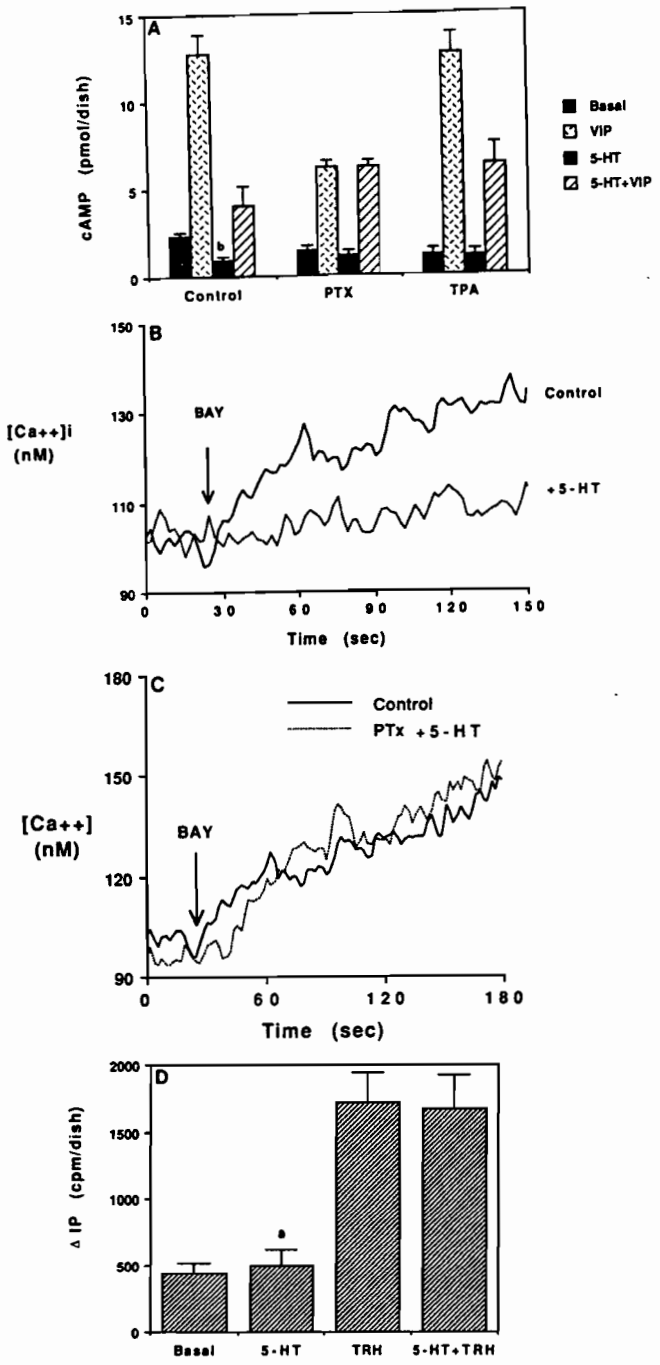

FIG. 1. **5-HT1A receptor-mediated signal transduction in GH4ZD10 cells.** *A*, 5-HT-induced inhibition of cAMP accumulation in GH4ZD10 cells. cAMP accumulation was measured in media harvested from cells 20 min after addition of regulators. The error bars indicate S.D. of triplicate determinations from one experiment which was repeated two additional times with similar results. "*b*" designates $p < 0.05$ by Student's t test compared with basal levels; the significance of other data points is readily apparent. Concentrations used were: 5-HT, 100 nM; VIP, 200 nM; TPA, 100 nM. PTx (25 ng/ml) was added 24 h prior to experimentation and was absent during assay. *B*, Inhibition of (±)-Bay K8644-induced changes in $[Ca^{2+}]_i$ by 5-HT in GH4ZD10 cells. The data are representative of three independent experiments. $[Ca^{2+}]_i$ was measured using calcium indicator Fura-2, and (±)-Bay K8644 (200 nM) or 5-HT (100 nM) added as indicated. (±)-Bay K8644 increased $[Ca^{2+}]_i$ by 1.41 ± 0.08-fold basal level (mean ± S.D., $n = 3$). *C*, blockade of 5-HT action on (±)-Bay K8644 by 25 ng/ml PTx. Concentrations of 5-HT and (±)-Bay K8466 were as in *B*, and PTx treatment was as in *A*. The data are representative of three independent experiments. *D*, 5-HT1A receptor activation does not alter PI turnover in GH4ZD10 cells. Intracellular levels of [^{3}H]inositol and inositol phosphates were measured as described under "Methods." Error bars represent S.D. of triplicate samples, and one of three experiments is shown. "*a*" denotes $p > 0.05$ compared with basal level. TRH (100 nM) or 5-HT (1 μM) suppressed calcium channel opening and inhibited increase in $[Ca^{2+}]_i$.

Finally, activation of the 5-HT1A receptor in GH4ZD10 cells induced no changes in PI hydrolysis (Fig. 1*D*). This observation is consistent with the lack of effect on basal $[Ca^{2+}]_i$, since generation of IP_3 via PI turnover is associated with increase in $[Ca^{2+}]_i$ in several cell types (Berridge and Irvine, 1989; Berridge, 1987). In contrast, 100 nM TRH, which induces characteristic spike and plateau phases of increase in $[Ca^{2+}]_i$ (Albert and Tashjian, 1984), induced a 3.8 ± 0.41-fold enhancement of total IP formation. Coaddition of 1 μM 5-HT during the 15-min incubation did not alter the TRH-induced enhancement of PI turnover. Thus, 5-HT was without effect on PI turnover in GH4ZD10 cells.

In summary, when expressed in GH4C1 pituitary cells, the 5-HT1A receptor is coupled to multiple intracellular signaling pathways (*i.e.* inhibition of adenylylcyclase, inhibition of calcium influx) characteristic of those found in differentiated neurons where the receptor is endogenously expressed.

5-HT1A Receptor Activation Increases $[Ca^{2+}]_i$ in Fibroblast Cells—A stably transfected clone of mouse Ltk$^-$ fibroblasts, named LZD-7, which expresses high levels (1.9 pmol/mg of protein) of specific rat 5-HT1A ligand binding sites (Albert *et al.*, 1990), provided a second cell type to examine the signaling phenotype of the 5-HT1A receptor. 5-HT (1 μM) had no effect on $[Ca^{2+}]_i$ in untransfected Ltk$^-$ cells (Fig. 2*A*), which lack endogenous 5-HT receptors (Albert *et al.*, 1990). By contrast, 5-HT induced an immediate increase in $[Ca^{2+}]_i$ in transfected LZD-7 cells, which was comprised of two phases: an acute "spike" phase and a sustained "plateau" phase. During the initial spike phase, $[Ca^{2+}]_i$ was increased by 2.67 ± 0.32 ($n = 8$) times the basal level (Table I), was maximal within 10–15 s, and lasted 30–40 s. During the following plateau phase, $[Ca^{2+}]_i$ remained increased by 1.45-fold basal level (Table I) for over 10 min. The selective 5-HT1A receptor agonist, 8-OH-DPAT (Albert *et al.*, 1990; Frazer *et al.*, 1990), mimicked the action of 5-HT to increase $[Ca^{2+}]_i$ (not shown). The 5-HT1A receptor antagonist spiperone (2 μM) (Albert *et al.*, 1990; Frazer *et al.*, 1990) abolished the increase in $[Ca^{2+}]_i$ induced by 100 nM 5-HT (Fig. 2*B*). Spiperone did not alter basal $[Ca^{2+}]_i$ and the vehicle (0.1% dimethyl sulfoxide) did not alter basal $[Ca^{2+}]_i$ or the 5-HT effect on $[Ca^{2+}]_i$. These results indicate that the change in intracellular calcium induced by 5-HT is indeed due to stimulation of the transfected 5-HT1A receptors.

Since the 5-HT1A receptor is thought to act by coupling to PTx-sensitive G proteins (Frazer *et al.*, 1990), the effect of PTx on 5-HT-induced increase in $[Ca^{2+}]_i$ was examined. As shown in Fig. 2*C*, actions of 5-HT on $[Ca^{2+}]_i$ were abolished completely by 24-h pretreatment of LZD-7 cells with 10 ng/ml PTx. The inhibitory effect of PTx on LZD-7 cells lasted 4–5 days, indicating that G protein turnover in this cell line is less rapid than in GH4C1 cells (48).

The effect of 5-HT to increase $[Ca^{2+}]_i$ in LZD-7 cells was dose-dependent, with an EC_{50} of 3 nM (Fig. 2, *D* and *E*), identical to the K_D of 5-HT at the rat 5-HT1A binding site (Albert *et al.*, 1990; Fargin *et al.*, 1986). Similar EC_{50} values were observed for both the spike and plateau phases of 5-HT-induced change in $[Ca^{2+}]_i$ (Fig. 2*E*), suggesting that the same receptor-mediated pathway mediates both phases. Taken together, these results indicate that 5-HT1A receptor activation mediates the 5-HT-induced increase in $[Ca^{2+}]_i$ in LZD-7 cells

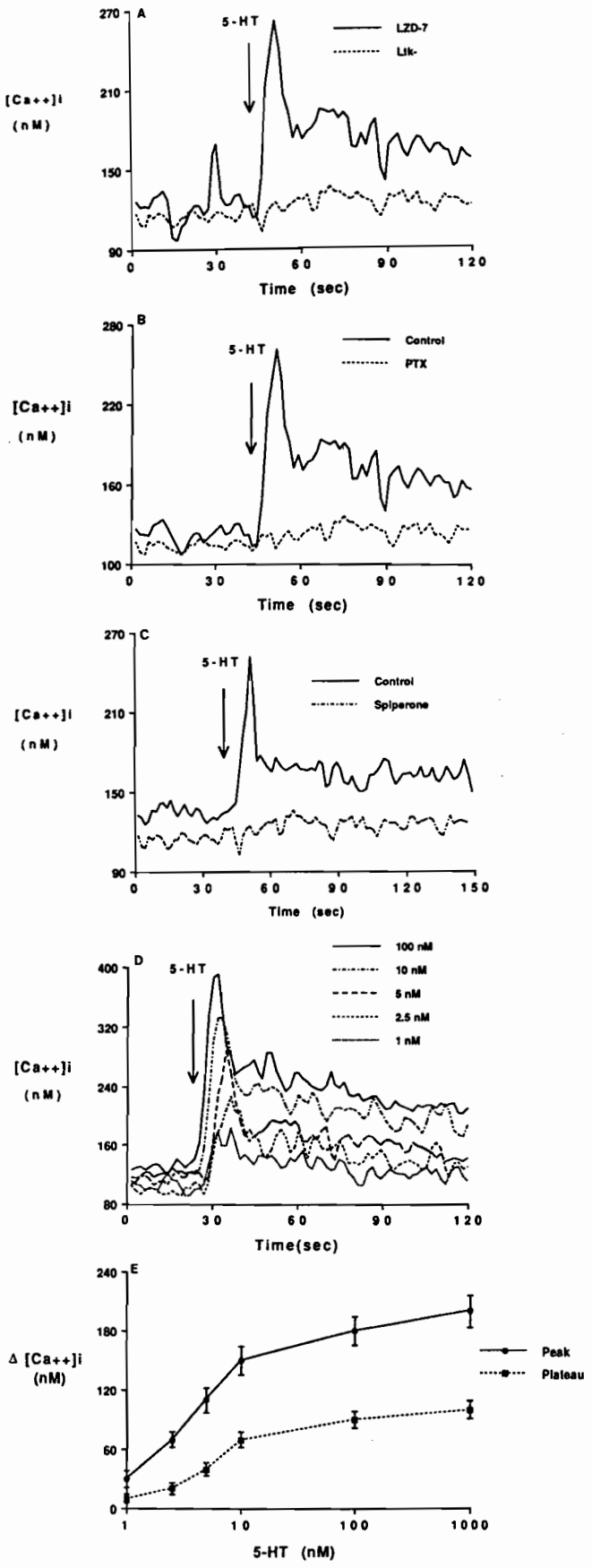

FIG. 2. **5-HT1A receptor activation increases in $[Ca^{2+}]_i$ in LZD-7 cells.** Changes in fluorescence intensity were monitored and converted to values of $[Ca^{2+}]_i$ as described under "Experimental Procedures." Curves generated by computer represent one of at least three independent experiments. *A*, 5-HT increases $[Ca^{2+}]_i$ in LZD-7 but not in Ltk⁻ cell. 5-HT (10 nM) was added to the cuvette as indicated. *B*, spiperone abolishes 5-HT-induced increase in $[Ca^{2+}]_i$ (control) in LZD-7 cells. 5 μM spiperone was added to the cell suspension as indicated. *C*, PTx blocks 5-HT-induced increase $[Ca^{2+}]_i$ in LZD-7 cells. PTx (10 ng/ml) was added to growth medium 24 h prior to assay. *D*, concentration dependence of 5-HT induced increase of $[Ca^{2+}]_i$ in LZD-7 cells. All curves were generated by a computer and were from a single experiment which has been repeated three times. *E*, dose-response curves for 5-HT-induced spike and plateau phases in increase of $[Ca^{2+}]_i$. Data were averaged from experiments in *D* and represent mean ± S.D. of at least five determinations.

TABLE I

Effect of protein kinases C and A on 5-HT-induced changes in $[Ca^{2+}]_i$

The influence of various acute pretreatments in LZD-7 cells on 100 nM 5-HT-induced changes in $[Ca^{2+}]_i$ are indicated as -fold basal level of $[Ca^{2+}]_i$ and represent mean ± S.D. of at least three independent determinations. A value of unity (1) indicates no change in the basal level of $[Ca^{2+}]_i$.

Treatment	5-HT-induced Increase in $[Ca^{2+}]_i$	
	Peak	Plateau
	-fold basal	
Control	2.67 ± 0.32	1.45 ± 0.21
TPA (1 nM)	2.42 ± 0.25[a]	1
TPA (10 nM)	1	1
Forskolin (10 μM)	2.13 ± 0.26[a]	1.34 ± 0.10[a]
8-Br-cAMP (1 mM)	2.21 ± 0.28[a]	1.28 ± 0.11[a]
Forskolin + 1 nM TPA	1	1
8-Br-cAMP + 1 nM TPA	1	1

[a] Indicates $p > 0.05$ compared with control values, *i.e.* no significant change.

via coupling to PTx-sensitive G proteins.

Sources of Calcium Mobilized by 5-HT—To characterize the sources of calcium for the 5-HT-induced increase in $[Ca^{2+}]_i$, LZD-7 cells were treated acutely with ionomycin, a calcium ionophore which releases cellular calcium stores (Albert and Tashjian, 1984, 1986). As shown in Fig. 3*A*, 100 nM ionomycin induced a 1.5-fold increase in $[Ca^{2+}]_i$ and nearly abolished the action of 5-HT added 2 min later. At a 10-fold higher concentration, ionomycin induced a 6-fold increase in $[Ca^{2+}]_i$, which returned to a near-basal plateau level (Fig. 3*B*). This concentration of ionomycin completely blocked 5-HT action. Since ionomycin did not cause a large sustained elevation in $[Ca^{2+}]_i$, it is likely that ionomycin preferentially depleted cellular calcium stores (Albert and Tashjian, 1984) and hence blocked 5-HT-induced calcium mobilization. Prior addition of 5-HT reduced by 30% the ionomycin-induced release of calcium (Fig. 3*C*), indicating that 5-HT partially releases ionomycin-sensitive calcium stores (Albert and Tashjian, 1986). Taken together, these data indicate that the major source of calcium for the 5-HT-induced changes is from release of ionomycin-sensitive intracellular stores, although a small component of calcium influx may be present. Since L cells lack voltage-dependent calcium channels (Liao *et al.*, 1990; Perez-Reyes *et al.*, 1990), it was not possible to test these cells for inhibition by 5-HT of (±)-Bay K8644-induced calcium influx.

5-HT Increases Hydrolysis of Phosphatidylinositol—The above results suggested that the change in $[Ca^{2+}]_i$ represented an IP_3-induced release of cellular calcium due to 5-HT1A receptor-mediated PI turnover, as seen for other receptors (Albert and Tashjian, 1984; Berridge and Irvine, 1989; Berridge, 1987). The actions of 5-HT on PI turnover in LZD-7 cells pre-equilibrated with [^{3}H]*myo*-inositol were examined. As shown in Fig. 4*A*, 5-HT increased total [^{3}H]inositol phosphate accumulation in a dose-dependent manner (EC_{50} = 5 nM). 5-HT-induced enhancement of PI turnover was mim-

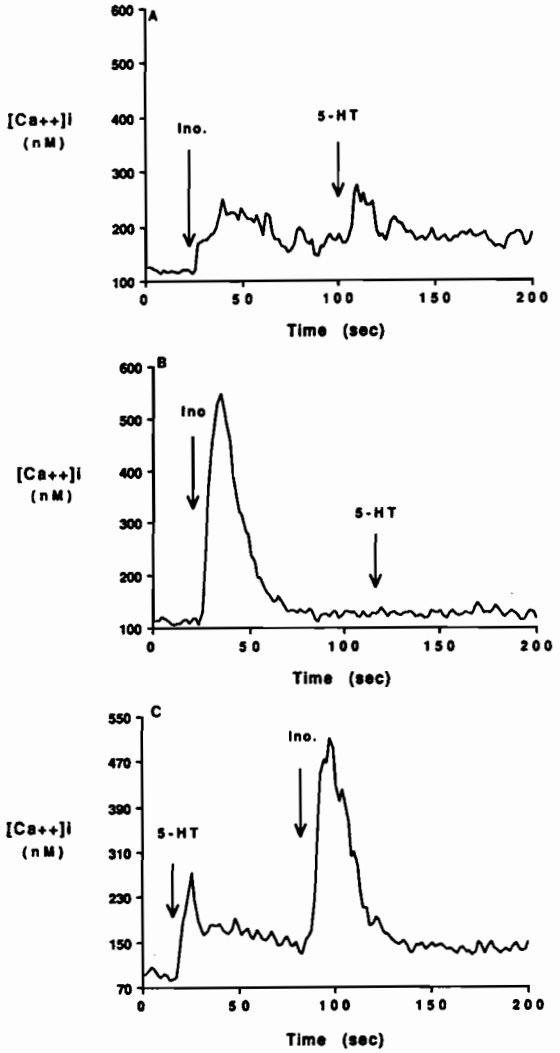

FIG. 3. **Sources of calcium for 5-HT-induced change in $[Ca^{2+}]_i$.** *A*, Ionomycin-induced increase in $[Ca^{2+}]_i$ and block of 5-HT induced changes. Ionomycin (*Ino.*, 100 nM) and 5-HT (100 nM) were added as indicated. Ionomycin (100 nM) induced an increase of $[Ca^{2+}]_i$ of 1.48 ± 0.10-fold basal level (mean ± S.D., $n = 3$). *B*, complete block of 5-HT-induced change in $[Ca^{2+}]_i$ by ionomycin (1 μM). Ionomycin (1 μM) induced a 6.3 ± 0.4-fold increase in basal $[Ca^{2+}]_i$ (mean ± S.D., $n = 3$). *C*, 5-HT was added prior to addition of ionomycin (1 μM). Following 5-HT, 1 μM ionomycin induced a 3.9 ± 0.2 ($n = 2$)-fold increase in $[Ca^{2+}]_i$. 10 nM 5-HT was added as indicated.

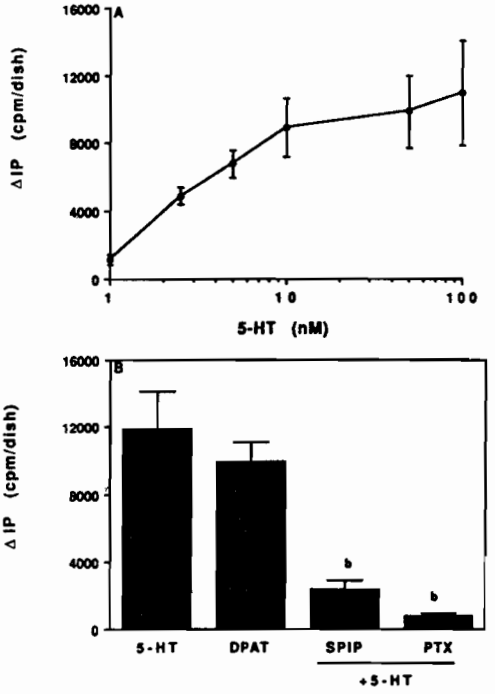

FIG. 4. **5-HT1A receptor activation enhances PI turnover in LZD-7 cells.** The *ordinate* values in each part represent (mean treated-mean untreated) values ± S.D. and are from at least three separate experiments. *A*, dose dependence of 5-HT-induced increase in inositol phosphate accumulation. Accumulation of $[^3H]IP_1$, $-IP_2$, and $-IP_3$ was measured as described (see "Experimental Procedures"). The basal $[^3H]IP$ production in untreated cells was 29,000 ± 3,000 cpm/dish. *B*, effect of various compounds on 5-HT-induced increase total PI turnover. The basal level of PI turnover was 30,000 ± 2,700 cpm/dish. PTx (10 ng/ml) was added 24 h before experimentation. The final concentrations of 5-HT and 8-OH-DPAT were 100 nM. Spiperone (*SPIP*, 5 μM) was added 5 min prior to addition of 5-HT and was present throughout the assay. "*b*" denotes a significant ($p < 0.05$) difference compared with 5-HT-treated controls.

icked by 1 μM 8-OH-DPAT and was blocked by 10 μM spiperone or by pretreatment with 10 ng/ml PTx (Fig. 4*B*), indicating that 5-HT1A receptor stimulates a phosphatidylinositol-specific phospholipase C via coupling to PTx-sensitive G proteins.

Although the increase (1.5-fold basal) in PI turnover induced by 5-HT in L cells was less pronounced than that induced by TRH in GH cells (Fig. 1*D*), the basal level of PI turnover under identical conditions was over 50-fold higher (*i.e.* 32,000 cpm *versus* 500 cpm). Thus the 5-HT-induced increment in PI turnover was actually over five times greater than with TRH in GH cells.

5-HT Inhibits Forskolin-stimulated cAMP Accumulation—Since the 5-HT1A receptor is coupled to inhibition of adenylylcyclase in other systems (Albert *et al.*, 1990; Clark *et al.*, 1987; Dumuis *et al.*, 1988; Fargin *et al.*, 1989; Okada *et al.*, 1989; Zgombick *et al.*, 1989), the effect of 5-HT on cAMP accumulation in media from LZD-7 cells was determined. As shown in Fig. 5*A*, 5-HT had no effect on basal cAMP accumulation under the present experimental conditions. However, the increase in cAMP accumulation (*i.e.* 4-fold above basal) induced by forskolin, which directly stimulates adenylylcyclase, was decreased by up to 60% in the presence of 0.01–1 μM 5-HT (EC_{50} = 10 nM, Fig. 5*B*). The effect of 5-HT to decrease forskolin-stimulated cAMP accumulation was mimicked by 8-OH-DPAT and blocked by spiperone or by pretreatment with 10 ng/ml PTx (Fig. 5*A*).

Selective Blockade of 5-HT Actions by Activation of Protein Kinases C and A—Receptor-mediated activation of phospholipase C produces DAG, an endogenous stimulator of protein kinase C (Nishizuka, 1986, 1988). Activation of protein kinase C exerts feedback inhibition of phospholipase C in several systems (Bell *et al.*, 1985; Brock *et al.*, 1985; Daniel-Issakani *et al.*, 1989; Rink *et al.*, 1983; Smith *et al.*, 1987; Ueda *et al.*, 1989, Van Corven *et al.*, 1989), including fibroblast cells (Brown *et al.*, 1987; Mendoza *et al.*, 1986). TPA, which mimics the action of DAG to stimulate protein kinase C activity (Castagna *et al.*, 1982; Nishizuka, 1986), was used to examine the importance of protein kinase C in 5-HT1A receptor signaling. LZD-7 cells were pretreated with various concentrations of TPA 1 min prior to addition of 5-HT (Fig. 6). Although TPA alone did not alter $[Ca^{2+}]_i$, the 5-HT-induced increase in $[Ca^{2+}]_i$ was abolished in a dose-dependent manner by acute pretreatment with 1–10 nM TPA, in multiple experiments (Table I). Interestingly, the two phases of change in $[Ca^{2+}]_i$ induced by 5-HT had different sensitivities to TPA. Following addition of 1 nM TPA, the plateau phase was almost

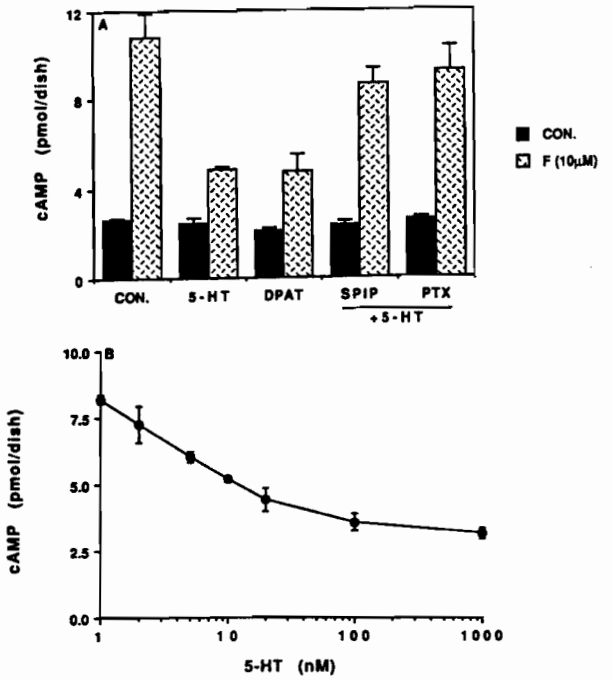

FIG. 5. **5-HT1A receptor activation inhibits adenylylcyclase activation in LZD-7 cells.** *A*, effects of various compounds on 5-HT actions on cAMP accumulation. PTx (10 ng/ml) was added 24 h before experimentation. Drug concentrations were as in Fig. 4. *B*, dose dependence of 5-HT-induced inhibition of forskolin (*F*)-stimulated cAMP levels in LZD-7 cells. Values in each part are the mean ± S.D. of results from three separate experiments.

totally blocked; the spike phase was less affected. However, in the presence of 10 nM TPA, the spike phase was also blocked (Fig. 6*B*). These results indicates that the 5-HT-induced increase in $[Ca^{2+}]_i$ consists of two distinct phases with different sensitivities to protein kinase C.

In order to investigate the role of cAMP in the 5-HT-induced increase of $[Ca^{2+}]_i$, and the interrelationship between protein kinase C and protein kinase A, the actions of forskolin on the 5-HT-induced increase in $[Ca^{2+}]_i$ and on the inhibitory actions of protein kinase C were examined. As shown in Fig. 6*C*, pretreatment with 10 μM forskolin had no significant effect on the 5-HT-induced increase in $[Ca^{2+}]_i$ and only partially (20–30%) inhibited the spike phase (Table I). Although increase in cAMP levels did not greatly alter the 5-HT-induced change in $[Ca^{2+}]_i$, it did synergize with TPA to block the change in $[Ca^{2+}]_i$. After pretreatment of the cells with 10 μM forskolin for 5 min, the 5-HT-induced increase in $[Ca^{2+}]_i$ was completely abolished by 1 nM TPA (Fig. 6*C*), whereas 1 nM TPA alone only partially-blocked the 5-HT response. The above actions of forskolin were mimicked by treatment with 10 mM 8-Br-cAMP (Fig. 6*D*, Table I), a nonhydrolyzable analog of cAMP, indicating that activation of protein kinase A mediates the forskolin-induced potentiation of protein kinase C actions on $[Ca^{2+}]_i$. Prior down-regulation of protein kinase C using 24–48-h pretreatment with 500 nM TPA blocked acute inhibition of 5-HT-induced increase in $[Ca^{2+}]_i$ by TPA alone or TPA plus forskolin (not shown), suggesting that protein kinase C is essential for these responses.

To study whether action of protein kinase C on calcium mobilization could be mediated via inhibition of phospholipase C, the effect of TPA on total PI turnover in LZD-7 cells was tested. As shown in Fig. 7*A*, at 1 and 10 nM concentrations, TPA had a small (less than 10% above basal level) but not significant effect on basal IP production in LZD-7 cells.

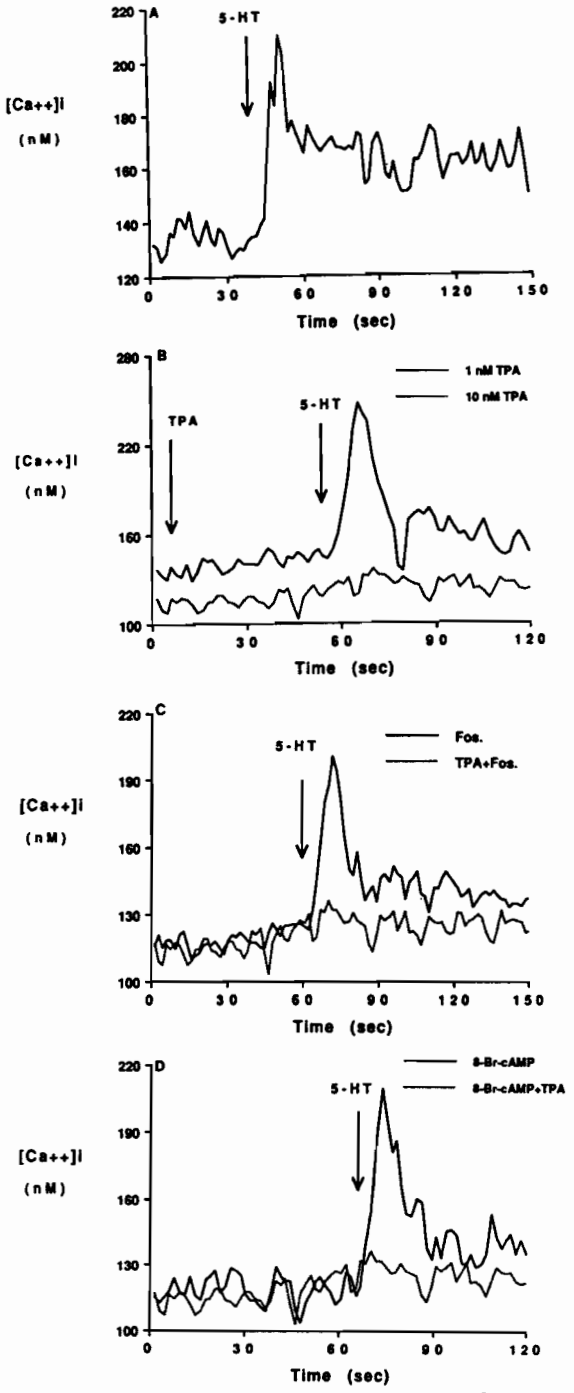

FIG. 6. **TPA blocks 5-HT-induced increase $[Ca^{2+}]_i$ in LZD-7 cells.** Curves represent a typical experiment which has been repeated at least five times with similar results. *A*, control: 10 nM 5-HT-induced increase in $[Ca^{2+}]_i$. *B*, blockade by 1 and 10 nM TPA of 5-HT-induced increases $[Ca^{2+}]_i$ in LZD-7 cells. TPA was added as indicated. *C*, forskolin (*Fos.*) potentiated the action of TPA on 5-HT-induced increase $[Ca^{2+}]_i$ in LZD-7 cells. LZD-7 cells were pretreated 10 μM forskolin for 5 min. 1 nM TPA and 10 nM 5-HT were added as indicated. *D*, 8-Br-cAMP (10 mM) potentiates the action of TPA (1 nM) to block the 5-HT-induced increase $[Ca^{2+}]_i$ in LZD-7 cells. 8-Br-cAMP was added 6 min prior to 5-HT.

90% only at 10 nM and had no significant effect at 1 nM (Fig. 7, legend). Forskolin alone altered neither basal nor 5-HT-induced increase in total PI turnover, indicating that activation of protein kinase A had no direct effect on phospholipase C activity. In the presence of 10 μM forskolin, 1 nM TPA now also inhibited 5-HT action effectively. These results corre-

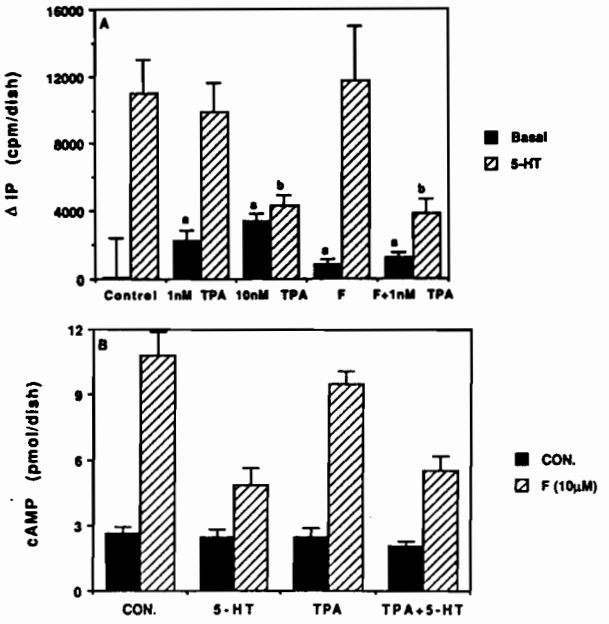

FIG. 7. **Actions of TPA on 5-HT-induced changes in cAMP and PI turnover.** *A*, TPA blocks the 5-HT-induced increase of total PI turnover. Concentrations used were: 5-HT, 10 nM; 10 μM forskolin (*F*); TPA, as indicated. The data for accumulation of [^{3}H]IP accumulation are presented as means ± S.D. ($n = 3$), with the basal level (32,000 ± 2270 cpm/dish) subtracted. "*a*" denotes $p > 0.05$ compared with control basal level; "*b*" denotes a significant difference ($p < 0.05$) compared with control 5-HT-treated samples. *B*, TPA does not alter 5-HT-induced decrease of forskolin (*F*)-induced cAMP levels. Concentrations used were: forskolin (*F*), 10 μM; 5-HT, 10 nM; and TPA, 10 nM. In both parts, the indicated compounds (*e.g.* 5-HT, TPA, or forskolin) were added acutely during the assay. Values in each part are the mean ± S.D. from three separate experiments.

induced enhancement of $[Ca^{2+}]_i$, indicating that activation of protein kinase C inhibits the 5-HT action at or prior to the activation of phospholipase C. The action of protein kinase A is indirect and requires simultaneous activation of protein kinase C.

We also tested whether activation of protein kinase C could block 5-HT-induced inhibition of cAMP accumulation to indirectly modulate protein kinase A. 10 nM TPA, which completely blocked 5-HT-induced increase in $[Ca^{2+}]_i$ (Fig. 6*B*), altered neither the basal level of cAMP nor forskolin-stimulated cAMP accumulation (Fig. 7*B*). Similarly, TPA had no effect on inhibition of forskolin-induced cAMP accumulation by 5-HT, indicating that this pathway is insensitive to protein kinase C activation, in contrast to the 5-HT-induced increase in $[Ca^{2+}]_i$, which is modulated by protein kinase C. Since 5-HT had no effect on the basal level of cAMP, the 5-HT-induced increase in $[Ca^{2+}]_i$ was not due to alteration of protein kinase A activity. Thus, TPA induced a rapid and differential uncoupling of 5-HT1A receptor action, inhibiting phospholipase C and calcium mobilization without altering 5-HT-induced inhibition of cAMP.

DISCUSSION

Phenotypic Switch of 5-HT1A Signal Transduction Pathway—Previous studies have shown that the 5-HT1A receptor is a member of the conserved family of receptors that interact with PTx-sensitive G proteins to inhibit adenylylcyclase activity (Frazer *et al.*, 1990). The 5-HT1A receptor is prominently expressed in neuronal cells (*e.g.* hippocampal CA-1, dorsal raphé nuclei) where it also opens potassium channels via activation of a PTx-sensitive G protein (Colino and Halliwell, 1987; Hoyer *et al.*, 1986; Ropert, 1988; Sprouse and Aghajanian, 1988; Zgombick *et al.*, 1989) and closes calcium channels (Pennington and Kelly, 1990; Ropert, 1988). This results in hyperpolarization of the membrane potential, closing of voltage-dependent calcium channels, and decrease in $[Ca^{2+}]_i$. A variety of other neurotransmitter receptors direct the opening of the G protein-gated potassium channel (including dopamine-D_2), each by activating a PTx-sensitive G protein (Birnbaumer *et al.*, 1990). As illustrated in Fig. 8, expression of the rat 5-HT1A receptor in pituitary GH4C1 cells (GH4ZD10 cells) resulted in a 5-HT-induced inhibition of $[Ca^{2+}]_i$ and cAMP accumulation similar to that observed in neurons. However, when expressed in Ltk$^-$ fibroblast cells (LZD-7 cells), activation of the 5-HT1A receptor caused a striking increase in $[Ca^{2+}]_i$, characteristic of receptors that induce PI turnover. In the same cells, 5-HT inhibited forskolin-stimulated cAMP accumulation in LZD-7 cells by over 50%. However, unlike in GH cells, 5-HT did not inhibit basal cAMP levels in LZD-7 cells. Thus, in L cells, the 5-HT1A receptor mediates a reversed intracellular signaling phenotype to induce both a dominant phospholipase C-mediated stimulatory pathway, as well as inhibitory intracellular actions.

The evidence that the 5-HT1A receptor mediated both stimulatory and inhibitory responses in LZD-7 cells is summarized here. 5-HT-induced increases in PI hydrolysis, in $[Ca^{2+}]_i$, and inhibition of cAMP accumulation in LZD-7 cells were: 1) mimicked by 8-OH-DPAT, a selective 5-HT1A receptor agonist, and blocked by spiperone, a 5-HT1A receptor antagonist; 2) dose-dependent, with potencies (EC_{50} = 3, 5, and 10 nM, respectively) that correlated well with receptor affinity of the 5-HT1A receptor for 5-HT (K_D = 1.7 nM) (Albert *et al.*, 1990; Frazer *et al.*, 1990); 3) abolished by pretreatment with PTx, which uncouples the receptor from G proteins, shifting the receptor from a high affinity state to a low affinity state (Albert *et al.*, 1990). This last-mentioned result indicates that all actions of the 5-HT1A receptor expressed in L cells were mediated via coupling to PTx-sensitive G proteins. Furthermore, both 5-HT-induced PI turnover and increase in $[Ca^{2+}]_i$ (but not inhibition of cAMP accumulation) were blocked by TPA-induced activation of protein kinase C. Thus, the 5-HT1A receptor appears to activate a phosphatidylinositol-specific phospholipase C to increase IP3 and $[Ca^{2+}]_i$ in LZD-7 cells via coupling to a PTx-sensitive signal transduction pathway that is distinct from the inhibitory neuroendocrine pathway and is subject to feedback inhibition by activation of protein kinase C.

The difference in 5-HT1A receptor signaling does not appear to be an artifact of overexpression of the receptor, whereby the 5-HT1A receptor is expressed at such high levels

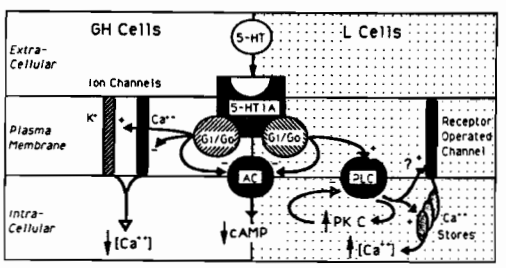

FIG. 8. **Model of the signal transduction pathways of the 5-HT1A receptor.** 5-HT binds to the 5-HT1A receptor and inhibits adenylylcyclase in both GH and L cells. In GH cells, 5-HT inhibits activation of Ca^{2+} channels and may (by analogy with neurons) open K^+ channels, leading to decrease in $[Ca^{2+}]_i$. In L cells, 5-HT activates PLC to release calcium stores and possibly activate receptor operated channels, to increase $[Ca^{2+}]_i$, and activate protein kinase C (*PK C*) via release of DAG. Protein kinase C negatively regulates PLC activation. Abbreviations are as in text except G_i/G_o, PTx-sensitive G proteins; *AC*, adenylylcyclase; *PK C*, protein kinase C.

in LZD-7 cells that an abnormal coupling to PLC results. The level of expression of 5-HT1A receptors in LZD-7 cells (1.9 pmol/mg of protein) is comparable with the expression in GH4ZD10 cells (1.1 pmol/mg) and is similar to the estimated level for expression in the CA1 region of the hippocampus (0.5 pmol/mg) (Albert *et al.*, 1990; Hoyer *et al.*, 1986). At a half-maximal concentration, which would activate 1.0 pmol/mg protein of 5-HT1A receptors, 5-HT induced a clear increase in PI turnover and in $[Ca^{2+}]_i$ in LZD-7 cells, but no increase in these parameters was observed in GH4ZD10 cells at maximal concentrations of 5-HT (Figs. 1 and 4). Thus, the activation of similar numbers of 5-HT1A receptors in different cell types produces different responses.

Recently, using a cloned human 5-HT1A receptor, it was shown that this receptor stimulates phospholipase C in HeLa cells, but not in COS-7 kidney cells (Fargin *et al.*, 1989; Raymond *et al.*, 1989). In HeLa cells, activation of PI turnover by 5-HT was regarded as a "weaker accessory signal pathway" to stimulate sodium-dependent phosphate uptake (Raymond *et al.*, 1989), since the EC_{50} for PI turnover was 10-fold higher than for inhibition of adenylylcyclase. In L cells, the actions of 5-HT to increase PI turnover and $[Ca^{2+}]_i$ appear to be primary actions of the 5-HT1A receptor, since the EC_{50} values for these actions range near to 5-HT binding affinity. Closely analogous observations have been reported for the dopamine-D_2 receptor, which is also coupled to PTx-sensitive G proteins to inhibition of $[Ca^{2+}]_i$ and cAMP in neurons. When expressed in L cells, the dopamine-D_2 receptor increases PI turnover and $[Ca^{2+}]_i$ that are blocked by pretreatment with PTx (Vallar *et al.*, 1990). Thus, the phenotypic switch from inhibition to excitation, which we observe with the 5-HT1A receptor, is not peculiar to this receptor alone but may be a general property of receptors which couple to PTx-sensitive G proteins. Expression of the stimulatory pathway seems to correlate with the differentiation state, in that rapidly growing tumor cell lines (*e.g.* L or HeLa cells) express the stimulatory fibroblast pathway, whereas cells (*e.g.* GH4C1 or neurons) which express differentiated properties (hormone production, excitability) express the inhibitory neuroendocrine pathway. Switching of receptor signaling phenotype would alter cellular regulation radically and may trigger changes in the differentiation state of the cell.

Mechanism of Change in Calcium Levels—As in other systems (Clark *et al.*, 1987; Dumuis *et al.*, 1988; Fargin *et al.*, 1989; Zgombick *et al.*, 1989), the 5-HT1A receptor in LZD-7 cells inhibited forskolin-stimulated adenylylcyclase activity, but had no effect on basal activity. We found no evidence for stimulation of adenylylcyclase by 5-HT1A receptor activation in L cells or in GH4C1 cells, as reported in some neurons (Dumuis *et al.*, 1988). Since 5-HT had no effect on the basal accumulation of cAMP, we conclude that cAMP is not the second messenger which mediates 5-HT-induced mobilization of intracellular calcium stores. Instead, 5-HT-induced increase of $[Ca^{2+}]_i$ were correlated with the 5-HT-induced enhancement of PI turnover.

The phosphoinositide signal transduction mechanism leads to production of two messengers, DAG and IP_3, each of which independently or together modulate many cellular responses in the number of cell types (Berridge and Irvine, 1989; Berridge, 1987). In many signaling systems, evidence has implicated 1,4,5-IP_3 as the molecule which links receptor-activated PI turnover to transient mobilization of calcium from intracellular stores (Prentki *et al.*, 1984; Schulz *et al.*, 1989; Smith *et al.*, 1987; Walker *et al.*, 1987), resulting in a transient spike in $[Ca^{2+}]_i$ (Berridge and Irvine, 1989; Berridge, 1987) as observed with 5-HT in LZD-7 cells. The results with ionomycin (Fig. 3) suggest that ionomycin-releasable calcium stores may account for both acute and sustained actions of 5-HT on $[Ca^{2+}]_i$, since ionomycin blocked both phases of 5-HT action.

Modulation by Protein Kinases C and A—We found that 5-HT-induced changes in PI turnover and $[Ca^{2+}]_i$ in LZD-7 cells, but not changes in cAMP accumulation, were blocked by 1–2-min pretreatment with TPA, an activator of protein kinase C. This indicates that signaling of PI turnover by the 5-HT1A receptor is under negative feedback regulation, since the product, DAG, can inhibit its own production via activation of protein kinase C. Other cellular systems which display this sort of feedback inhibition include 3T3 (Brown *et al.*, 1987; Mendoza *et al.*, 1986) and human fibroblast cells (Van Corven *et al.*, 1989), smooth muscle cells (Brock *et al.*, 1985), and platelets (Bell *et al.*, 1985; Rittenhouse and Sasson, 1985). In each case, the receptor is coupled via a PTx-sensitive G protein to PI turnover.

There is evidence that TPA acts by blocking the coupling of PTx-sensitive G proteins to PLC (Ryu *et al.*, 1990; Smith *et al.*, 1987), either by phosphorylation of the G protein (Daniel-Issakani *et al.*, 1989) or of phospholipase C (Ryu *et al.*, 1990). Reconstitution experiments utilizing purified G_i proteins have demonstrated that these proteins do couple to phospholipase C (Ueda *et al.*, 1989). Activation of protein kinase C by TPA leads to phosphorylation of a specific PTx-sensitive G protein, G_i2, to inhibit insulin-like growth factor I receptor-mediated PIP_2 turnover (Drummond, 1985). A similar mechanism may mediate the action of TPA to block 5-HT1A receptor-induced PI turnover. By transfecting antisense G_i2, we have evidence that the 5-HT1A receptor is coupled via G_i2 to enhance $[Ca^{2+}]_i$ in LZD-7 cells.[2] Recently, it has become apparent that different PTx-sensitive G proteins may link to different signal transduction pathways (Birnbaumer *et al.*, 1990; Ross, 1989). For example, G_i3 couples the somatostatin receptor to potassium channels (Yatani *et al.*, 1987), G_i2 and G_o couple couple to activation of PI turnover in *Xenopus* oocytes (Moriarty *et al.*, 1990). Understanding the specificity of coupling of receptors to G protein in different cellular models will be a key step to understanding why receptors possess different signal transduction pathway in different cell types.

A novel finding was the potentiation of TPA action by activators of protein kinase A. Inhibition (Sibley *et al.*, 1984) or enhancement (Bell *et al.*, 1985; Cronin and Canonico, 1985) of cAMP generation by activation of protein kinase C has been reported, but little is known about actions of protein kinase A on protein kinase C (*e.g.* McAtee and Dawson, 1989), particularly in modulating signal transduction. The present results indicate that activators of the adenylylcyclase pathway can enhance C kinase-mediated homologous uncoupling of receptors in the phospholipase C pathway.

Conclusion—We have shown that the signal transduction pathway of the 5-HT1A receptor depends on the cell type in which it is expressed. In fibroblast L cells, the receptor couples efficiently to phospholipase C to increase $[Ca^{2+}]_i$, due to both influx of extracellular calcium and release of stored intracellular calcium. This represents an altered phenotype of 5-HT1A receptor signaling, where inhibition of $[Ca^{2+}]_i$ is changed to stimulation. We have also shown that stimulation of $[Ca^{2+}]_i$ by 5-HT is rapidly and potently blocked by activation of protein kinase C (potentiated by protein kinase A), whereas the inhibitory branch (inhibition of adenylylcyclase) is not. Thus, the phenotypic switch of the receptor is accompanied by an altered regulation of receptor coupling. The role of a switch in signaling phenotype of this and other receptors

[2] Y. F. Liu and P. R. Albert, unpublished observations.

in vivo remains an open question. However, evidence relating activation of phospholipase C and protein kinase C to the growth-promoting actions of oncogenes and growth factors (Berridge, 1987; Mendoza *et al.*, 1986; Weinstein, 1988) point to a possible role of the phenotypic switch in regulation of precursor cell growth and differentiation.

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CHAPTER IV CHOLERA TOXIN-SENSITIVE 3',5'-CYCLIC ADENOSINE MONOPHOSPHATE AND CALCIUM SIGNALS OF THE HUMAN DOPAMINE-D1 RECEPTOR: SELECTIVE POTENTIATION BY PROTEIN KINASE A

Cholera Toxin-Sensitive 3′,5′-Cyclic Adenosine Monophosphate and Calcium Signals of the Human Dopamine-D1 Receptor: Selective Potentiation by Protein Kinase A

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The signal transduction pathways of the dopamine-D1 receptor were investigated in two cell types stably transfected with the human D1 receptor cDNA, rat pituitary GH4C1 cells (GH4-hD1), and mouse Ltk− fibroblast cells (L-hD1). In both GH4-hD1 and L-hD1 cell lines, stimulation of the dopamine-D1 receptor induced a marked increase in cAMP accumulation. In addition, dopamine potentiated activation of L-type voltage-dependent calcium channels in a cAMP-dependent manner in GH4-hD1 cells. However, in L-hD1 cells, dopamine increased cytosolic free calcium concentrations ($[Ca^{++}]_i$) by mobilization of intracellular calcium rather than by calcium influx. This effect was correlated with a dopamine-induced enhancement of phospholipase C activity in L-hD1 cells. Pretreatment (24 h) with cholera toxin (CTX) was used to maximally activate the GTP-binding protein (G protein) Gs, causing a maximal elevation of cAMP levels and uncoupling the D1 receptor from Gs. The described actions of dopamine in both cell lines were abolished by pretreatment with CTX, indicating that CTX substrates (*e.g.* Gs) may mediate these actions. The blockade by CTX was not due to CTX-induced elevation of cAMP, since pretreatment with forskolin or 8-bromo-cAMP to activate cAMP-dependent protein kinase did not inhibit dopamine actions nor alter basal $[Ca^{++}]_i$. Pretreatment (1–3 h) of L-hD1 cells with forskolin (10 μM) or 8-bromo-cAMP (5 mM) altered neither the basal activity of phospholipase C nor basal $[Ca^{++}]_i$ in L-hD1 cells but greatly enhanced the dopamine-induced increase of phosphatidyl inositol turnover and $[Ca^{++}]_i$. From these results we conclude that: 1) the dopamine-D1 receptor induces multiple and cell-specific signals, including elevation of cAMP levels in both GH and L cells, cAMP-dependent activation and potentiation of opening of L-type voltage-dependent calcium channel in GH cells, and a novel phosphatidyl inositol-linked mobilization of cellular calcium in L cells; 2) coupling of the D1 receptor to these responses involves CTX-sensitive proteins, possibly Gs; and 3) acute preactivation of cAMP-dependent protein kinase can markedly enhance, rather than attenuate, certain pathways of dopamine-D1 transmembrane signaling. (Molecular Endocrinology 6: 1815–1824, 1992)

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INTRODUCTION

The cloned human dopamine-D1 receptor has structural homology to members of a family of hormone and neurotransmitter receptors which share the common structural feature of seven putative membrane-spanning domains. These receptors couple to GTP-binding proteins (G proteins, *e.g.* Gs) which activate effectors [such as adenylyl cyclase (AC)] to generate intracellular signals (*e.g.* elevation of cAMP levels) (1, 2). Consistent with the diversity of receptors, G proteins, and effectors (1–4), it has been demonstrated that individual receptors can induce multiple signal transduction pathways (1, 5–7). Receptors with dopamine-D1 pharmacology have been shown to couple to activation of AC in membranes prepared from several tissues (8–10), presumably via coupling to Gs. In addition, D1-agonists have been shown to enhance phosphatidyl inositol (PI) turnover (11–13). However, with the discovery of multiple dopamine receptors with overlapping pharmacology (14), a key question is whether a single subtype of D1 receptor can activate both cAMP and PI pathways, or whether separate receptors activate these responses. As a first step to addressing this question, it

is necessary to characterize the functional properties of individual dopamine receptor subtypes in isolation.

Using heterologous expression of cloned receptor subtypes in homogenous, receptor-negative cell lines allows determination of the full range of signaling properties of single receptor subtypes in selected cellular environments (*e.g.* 2, 5, 6). We have used this approach to investigate whether the dopamine-D1 receptor cDNA encodes a functional receptor, which signal transduction pathways the receptor regulates, and how activation of cAMP-dependent protein kinase modulates these pathways. Two different cell lines, rat pituitary GH4C1 cells and mouse Ltk− cells, were transfected with the cloned human dopamine-D1 receptor (15) to generate GH4-hD1 and L-hD1 cell lines, respectively. The parental cell lines lack endogenous dopamine-D1 receptors and responses, and have been previously shown to mediate different types of receptor signal transduction (5, 6). In both GH4-hD1 and L-hD1 cells, the cloned dopamine-D1 receptor markedly enhanced cAMP accumulation. In addition, a cAMP-dependent activation of calcium channels mediated by the D1-receptor was observed in GH4-hD1 cells. In L-hD1 cells, the cloned human D1 receptor induced an acute increase in cytosolic free calcium levels ($[Ca^{++}]_i$) which was correlated with enhancement of phospholipase C (PLC) activity. To analyze the G proteins involved in these dopamine-induced responses, we pretreated the cells with cholera toxin (CTX) to maximally ADP-ribosylate and activate Gs (16–18). Once activated, Gs induced a large increase in cAMP and became uncoupled from the dopamine-D1 receptor. Thus we found that all responses to dopamine were abolished by the CTX pretreatment, suggesting mediation by Gs or a Gs-like protein. In L-hD1 cells, the blockade of dopamine action was not due to the elevation of cAMP induced by CTX, because elevation of cAMP using forskolin or 8-bromo-cAMP did not block the calcium response. On the contrary, both the dopamine-induced PI turnover and calcium signal were greatly enhanced by prior elevation of cAMP levels to activate cAMP-dependent protein kinase. Thus we conclude that the dopamine-D1 receptor, via Gs, can induce multiple and cell-specific signals in GH and L cells: the predominant changes include increase in cAMP levels in both cell types, a cAMP-dependent calcium signal in GH cells, and a novel and cAMP-independent calcium mobilization in L cells which is positively modulated by acute increase in cAMP levels and may become the predominant intracellular signal when cAMP-dependent protein kinase is preactivated. These actions of the receptor may play important roles in tissues where the receptor is expressed.

RESULTS

Dopamine-Induced Increase in cAMP Accumulation in L-hD1 and GH4-hD1 Cells

Both GH4-hD1 and L-hD1 cells exhibited specific, high affinity binding to [^{3}H]SCH 23390, a selective dopamine-D1 antagonist (9, 10). The maximal binding capacity was 0.20 ± 0.01 pmol/mg protein for GH4-hD1 cells and 1.6 ± 0.1 (n = 3) pmol/mg protein for L-hD1 cells, and the dissociation constant. was 0.3 nM for both cell lines. To examine whether the expressed human dopamine-D1 receptor in L-hD1 and GH4-hD1 cells is functionally coupled to activation of AC, as it is in brain (8), the effect of dopamine on cAMP accumulation was tested. As shown in Fig. 1A, 1 μM dopamine induced an 80-fold (L-hD1) or a 15-fold (GH4-hD1) increase in cAMP accumulation. The specific D1 receptor antagonist SCH 23390 (1 μM) completely blocked the action of dopamine, while spiperone or sulpiride (dopamine-D2 receptor antagonists) had no effect. The increase in cAMP accumulation in both L-hD1 and GH4-hD1 cells was concentration dependent (Fig. 1B), with EC_{50} values for dopamine of 12 ± 2 nM and 20 ± 3 nM (n = 3), respectively, similar to values found for activation of AC by dopamine in brain membrane preparations (8–10). Dopamine (1 μM) did not alter cAMP accumulation in nontransfected Ltk$^-$ and GH4C1 cells, nor in GH4ZD10 or LZD-7 cells, which are transfected with the 5-HT1A receptor in the pZEM-3 plasmid (data not shown). The above results indicate that in both GH and L cells, activation of the human dopamine-D1 receptor stimulates AC by coupling to Gs.

To assess which G protein mediated the dopamine-induced increase in cAMP accumulation, L-hD1 and GH4-hD1 cells were pretreated overnight with CTX (2 μg/ml) or pertussis toxin (PTX; 25 ng/ml). By ADP-ribosylating target G protein α-subunits, CTX constitutively activates α_s, while PTX inactivates α_i/α_o (16–19). Pretreatment with CTX increased cAMP accumulation in both L-hD1 and GH4-hD1 cells, and dopamine (1 μM) did not induce further increase in cAMP accumulation (Fig. 1C). In contrast, PTX pretreatment altered neither basal nor dopamine-stimulated cAMP accumulation in L-hD1 or GH4-hD1 cells. Thus, the dopamine-D1 receptor stimulates AC via a CTX-sensitive mechanism, presumably by coupling to Gs.

Dopamine-Induced Activation of L-Type Calcium Channels in GH4-hD1 Cells

The action of dopamine-D1 receptor activation on cytosolic free calcium concentration ($[Ca^{++}]_i$) in GH4-hD1 cells was determined by measuring fluorescence changes in cells loaded with the calcium-dependent fluorescent indicator fura-2 (6, 20). As shown in Fig. 2, dopamine (1 μM) consistently induced a gradual increase in $[Ca^{++}]_i$ in GH4-hD1 cells, which was maximal within 60 sec. In contrast, dopamine had no effect on $[Ca^{++}]_i$ in nontransfected GH4C1 or in GH4ZD10 cells (data not shown). In six independent experiments, dopamine induced a significant increase in $[Ca^{++}]_i$ of 1.23 ± 0.1-fold basal level (Table 1). By contrast, in cells pretreated with D1 antagonist 1 μM SCH 23390 (Fig. 2A), dopamine failed to induce any change in basal $[Ca^{++}]_i$. SCH 23390 alone induced no change in basal $[Ca^{++}]_i$ (not shown), and the diff

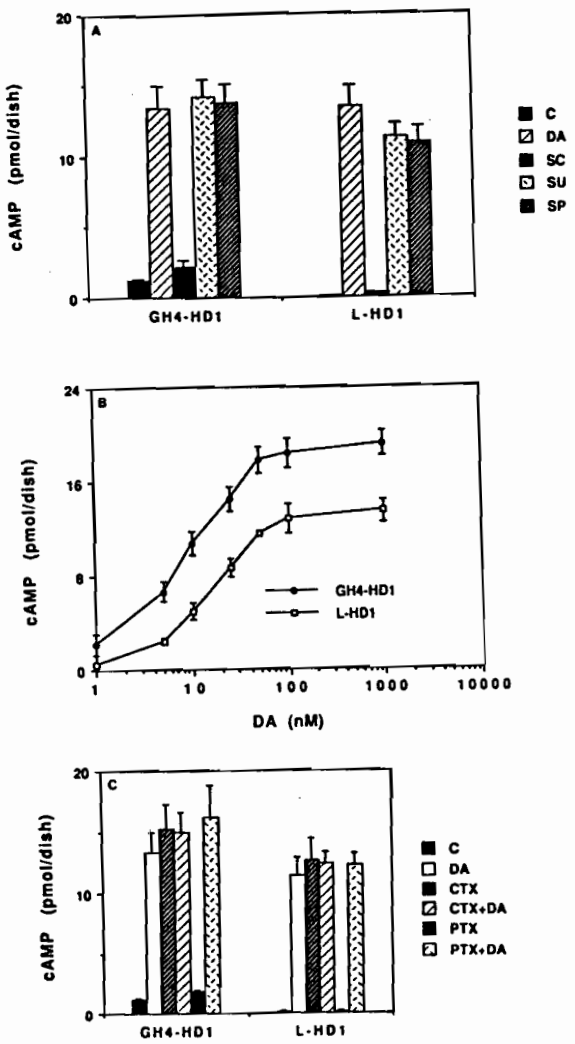

Fig. 1. Activation of the Dopamine-D1 Receptor Increases cAMP Accumulation in GH4-hD1 and L-hD1 Cells

A, Pharmacological profile of the dopamine-induced increase of cAMP accumulation in GH4-hD1 and L-hD1 cells. The values represent the means ± SD from three separate experiments done with triplicate samples. Dopamine was present in all samples (*i.e.* DA, SC, SU, and SP) except control samples (C), which were not treated. Concentrations used were: dopamine (DA), 1 μM; SCH 23390 (SC), 1 μM; sulpiride (SU), 10 μM; and spiperone (SP), 10 μM. B, Concentration dependence of the dopamine-induced increase of cAMP accumulation in GH4-hD1 and L-hD1 cells. The values are averages of three independent experiments each performed with triplicate determinations. C, Actions of CTX or PTX on dopamine-induced cAMP accumulation. CTX (2 μg/ml) or PTX (25 ng/ml) was added to the growth medium 24 h before experimentation and were absent during the assay. The control samples (C) were not treated with toxin or dopamine.

of the curves in Fig. 2 reflects the normal intersample variation of the fura-2 technique. In Fig. 2B, the addition of 1 μM nifedipine, an L-type calcium channel blocker (21), abolished the dopamine-induced increase in $[Ca^{++}]_i$, suggesting that the dopamine-induced increase in $[Ca^{++}]_i$ in GH4-hD1 cells was due mainly to opening L-type voltage-dependent calcium channels. Under the present conditions, nifedipine did not have a significant effect on basal $[Ca^{++}]_i$. Further evidence for the action of dopamine on L-type calcium channels was the potentiation by dopamine of the effect of (±)-Bay K8644, an L-type calcium channel agonist (21). The action of 100 nM (±)-Bay K8644 to increase $[Ca^{++}]_i$ was clearly enhanced by prior addition of 1 μM dopamine (Fig. 2C and Table 1). More detailed studies showed that acute dopamine pretreatment shifted the concentration dependence curve of (±)-Bay K8644 to the left, with a reduction in EC_{50} value from 300 nM to 40 nM (Fig. 2D). These findings indicate that dopamine-D1 receptor activation in GH4-hD1 cells markedly enhances the excitability of L-type calcium channels, promoting calcium influx in both basal and stimulated conditions.

To determine the role of cAMP in the dopamine-D1 receptor-mediated activation of calcium channels in GH4-hD1 cells, forskolin was used and shown to induce a nifedipine-sensitive gradual increase in $[Ca^{++}]_i$ in GH4-hD1 cells, as observed in nontransfected GH4C1 cells (22). Coaddition of dopamine did not lead to further increase in $[Ca^{++}]_i$ (Table 1), indicating a role for cAMP in dopamine-induced activation of calcium channels under basal conditions. Similarly, forskolin or the cAMP analog 8-bromo-cAMP mimicked the action of dopamine to enhance (±)-Bay K8644-induced increase in $[Ca^{++}]_i$. Coaddition of dopamine did not further enhance (±)-Bay K8644 (Table 1). Taken together, these data support the hypothesis that the dopamine-D1 receptor, via activation of cAMP-dependent protein kinase, indirectly enhances the opening of L-type voltage-dependent calcium channels in GH cells.

To examine which G protein is involved in the action of dopamine on $[Ca^{++}]_i$, GH4-hD1 cells were pretreated overnight with CTX. As shown in Fig. 2E, the action of dopamine was completely abolished by CTX pretreatment. Pretreatment with PTX did not inhibit the dopamine-induced increase in $[Ca^{++}]_i$. Therefore, Gs, but not Gi/Go, mediates the actions of the dopamine-D1 receptor to enhance calcium influx via L-type voltage-dependent calcium channels.

Dopamine-Induced Mobilization of Intracellular Calcium Stores in L-hD1 Cells

The effect of dopamine-D1 receptor activation on $[Ca^{++}]_i$ in L-hD1 cells was larger and had a different time course from that in GH4-hD1 cells, as shown in Fig. 3. In L-hD1 cells, but not in nontransfected Ltk− cells or the 5-HT1A receptor-expressing LZD-7 cells, dopamine initiated an immediate peak of increase in $[Ca^{++}]_i$ (1.45 ± 0.13-fold, n = 6), which decayed to a near-basal level (Fig. 3A). The increase in $[Ca^{++}]_i$ induced by dopamine was concentration dependent, with an EC_{50} of 120 ± 30 nM (Fig 3B), and was completely abolished by 1 μM SCH 23390 (Fig 3A). Alone, SCH 23390 did not significantly alter basal $[Ca^{++}]_i$. These results indicate that the dopamine-induced increase in $[Ca^{++}]_i$ in L-hD1 cells is due to D1 receptor activation

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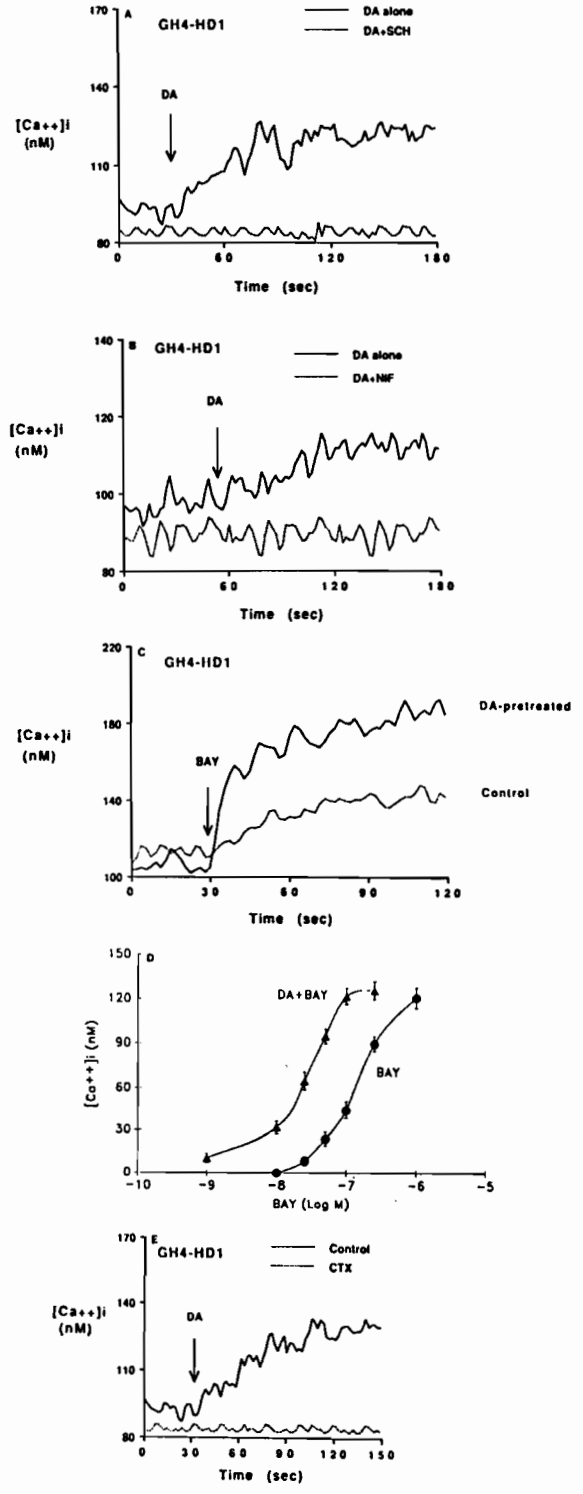

Fig. 2. Activation of L-Type Voltage-Dependent Calcium Channels by Dopamine in GH4-hD1 Cells

A, Dopamine-induced increase in $[Ca^{++}]_i$ in GH4-hD1 cells is blocked by D1-antagonist SCH 23390 (SCH). *Curves* generated by computer represent a typical experiment which was performed in duplicate and was repeated three times. The average increase in $[Ca^{++}]_i$ induced by 1 μM dopamine was 1.2 ± 0.21-fold above basal (see Table 1). SCH 23390 (1 μM) was added to the sample 1 min before recording. B, Nifedipine blocks the dopamine-induced increase in $[Ca^{++}]_i$ in GH4-hD1 cells. Nifedipine (NIF; 1 μM) was added to the cell suspension 5 min before recording. Nifedipine did not alter basal $[Ca^{++}]_i$ in the present condition. C, Dopamine enhances the (±)-Bay K8644-induced increase in $[Ca^{++}]_i$ in GH4-hD1 cells. The cells were pretreated with 1 μM dopamine for 3 min before recording. (±)-Bay K8644 (BAY; 100 nM) was added to both samples as indicated. The *curves* are representative of three separate experiments. D, Dopamine pretreatment increases potency of (±)-Bay K8644 action on $[Ca^{++}]_i$ in GH4-hD1 cells. Concentration dependence curve of (±)-Bay K8644 from GH4-hD1 cells treated (▲) or not treated (●) with dopamine (1 μM). The data are the average of three independent experiments. E, Pretreatment with CTX abolishes the dopamine-induced increase in $[Ca^{++}]_i$ in GH4-hD1 cells. CTX (2 μg/ml) was added to the growth media 24 h before experimentation, and dopamine (1

The distinct time courses of D1-induced calcium changes in GH and L cells suggested that different mechanisms may be involved. To test whether dopamine mobilizes intracellular calcium in L cells, we pretreated cells with ionomycin, a calcium ionophore which releases calcium from the intracellular stores, without altering receptor-induced calcium influx (23, 24). In L-hD1 cells, 100 nM ionomycin induced a small increase in $[Ca^{++}]_i$ of 1.48 ± 0.11-fold over basal level (n = 3). Subsequent addition of dopamine did not further increase $[Ca^{++}]_i$ (Fig. 3C), suggesting that the dopamine-induced increase in $[Ca^{++}]_i$ was mainly due to mobilization of intracellular ionomycin-sensitive calcium stores. By contrast, ionomycin did not block dopamine-induced increase in $[Ca^{++}]_i$ in GH4-hD1 cells (data not shown).

To analyze which G protein mediates the dopamine action on $[Ca^{++}]_i$, L-hD1 cells were pretreated for 24 h with CTX (2 μg/ml) or PTX (25 ng/ml) (Fig. 4). In CTX-pretreated cells, the action of dopamine was completely abolished (Fig. 4A). In contrast, activation of the endogenous P2-purinergic receptor with 1 μM ATP initiated a normal increase in $[Ca^{++}]_i$ in CTX-pretreated L-hD1 cells (Fig. 4B). Pretreatment (24 h) of L-hD1 cells with forskolin, to mimic the CTX-induced increase in cAMP levels, did not block the dopamine action on $[Ca^{++}]_i$, but actually enhanced it (see below). This result indi-

Table 1. Dopamine Potentiates BAY-Induced Activation of L-Type Calcium Channels in GH4-hD1 Cells

	Control	BAY (100 nM)
Basal	1.00	1.25 ± 0.15
DA (1 μM)	1.23 ± 0.10	1.98 ± 0.21
Forskolin (10 μM)	1.32 ± 0.11	2.01 ± 0.28[a]
8-Bromo-cAMP (5 mM)	ND	1.91 ± 0.15[a]
DA + forskolin	1.28 ± 0.13	1.94 ± 0.21[a]
DA + 8-bromo-cAMP	1.25 ± 0.11	1.89 ± 0.19[a]

The effects of various compounds on dopamine (DA) or (±)-Bay K8644 (BAY)-induced increase in $[Ca^{++}]_i$ (denoted as fold increase in basal $[Ca^{++}]_i$), are represented as means ± SD of at least three independent experiments. ND, Not determined.
[a] $P > 0.05$ as compared to the $[Ca^{++}]_i$ level induced by DA + BAY.

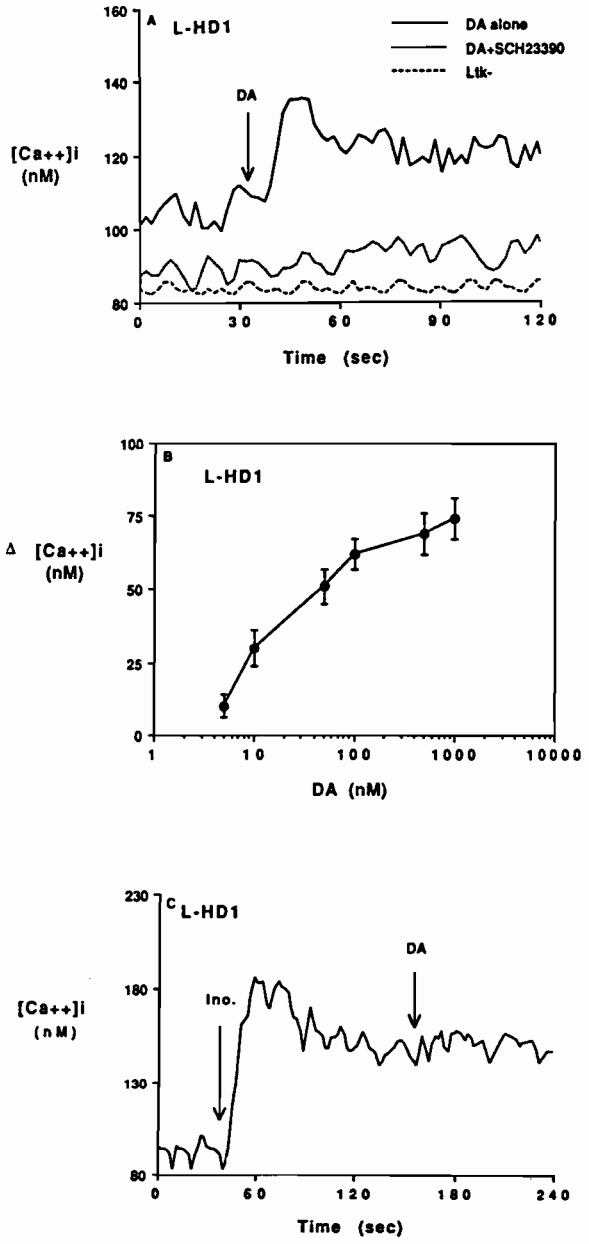

Fig. 3. Activation of the Dopamine-D1 Receptor Induces Mobilization of Intracellular Calcium Stores in L-hD1 Cells

Changes of $[Ca^{++}]_i$ in L-hD1 cells were measured in *Materials and Methods*. A, Dopamine (1 μM) induces increase in $[Ca^{++}]_i$ in L-hD1 cells but not in nontransfected Ltk− cells. The action of dopamine is abolished by SCH 23390 (1 μM). B, Concentration dependence curve for the peak and plateau level of the dopamine-induced increase in $[Ca^{++}]_i$ in L-hD1. The *error bars* are SD of three independent experiments performed in duplicate determinations. C, Blockade by ionomycin of the dopamine-induced increase in $[Ca^{++}]_i$ in L-hD1 cells. Ionomycin (Ino.; 100 nM) and dopamine (DA; 1 μM) were added as indicated.

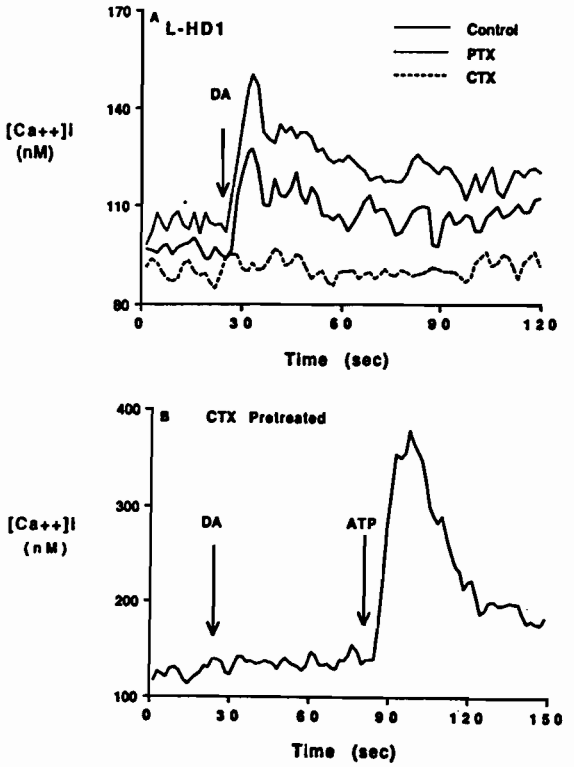

Fig. 4. Effect of CTX and PTX Pretreatment on the Calcium Signal Induced by Dopamine in L-hD1 Cells

Curves represent one of at least three independent experiments. Dopamine (1 μM) was added to each sample as indicated. A, Abolition by CTX but not PTX of the dopamine-induced increase in $[Ca^{++}]_i$ in L-hD1 cells. CTX (2 μg/ml) or PTX (10 ng/ml) was added to the growth medium 24 h before assay and were absent during assay. B, CTX pretreatment selectively blocks dopamine- but not ATP (200 μM)-induced increase in $[Ca^{++}]_i$ in L-hD1 cells. The average value of increase in $[Ca^{++}]_i$ induced by 200 μM ATP in untreated Ltk− cells was 4-fold over the basal level.

cates that blockade of dopamine action by CTX pretreatment was not mediated by elevation of cAMP levels. When L-hD1 cells were pretreated with PTX

$[Ca^{++}]_i$ was significantly enhanced (40 ± 6%, n = 4, $P < 0.05$) compared to untreated cells, suggesting that the Gs-mediated action of the dopamine-D1 receptor to mobilize intracellular calcium stores may be partially inhibited by Gi/Go in the quiescent condition. Taken together, these results indicate that a CTX-sensitive G protein (Gs, not Gi, Go, or Gplc) mediates the D1 receptor-induced calcium mobilization.

Positive Modulation by cAMP-Dependent Protein Kinase

As previously observed in L cells (6, 25), neither forskolin nor 8-bromo-cAMP altered $[Ca^{++}]_i$ in L-hD1 cells, suggesting that enhancement of cAMP levels does not mediate the dopamine-induced increase in $[Ca^{++}]_i$ in these cells. Thus, the dopamine-induced increase in $[Ca^{++}]_i$ in L-hD1 cells is cAMP independent. Surprisingly, pretreatment (1–3 h) of L-hD1 cells with forskolin (Fig. 5A) or 8-bromo-cAMP (Fig. 5B) markedly enhanced

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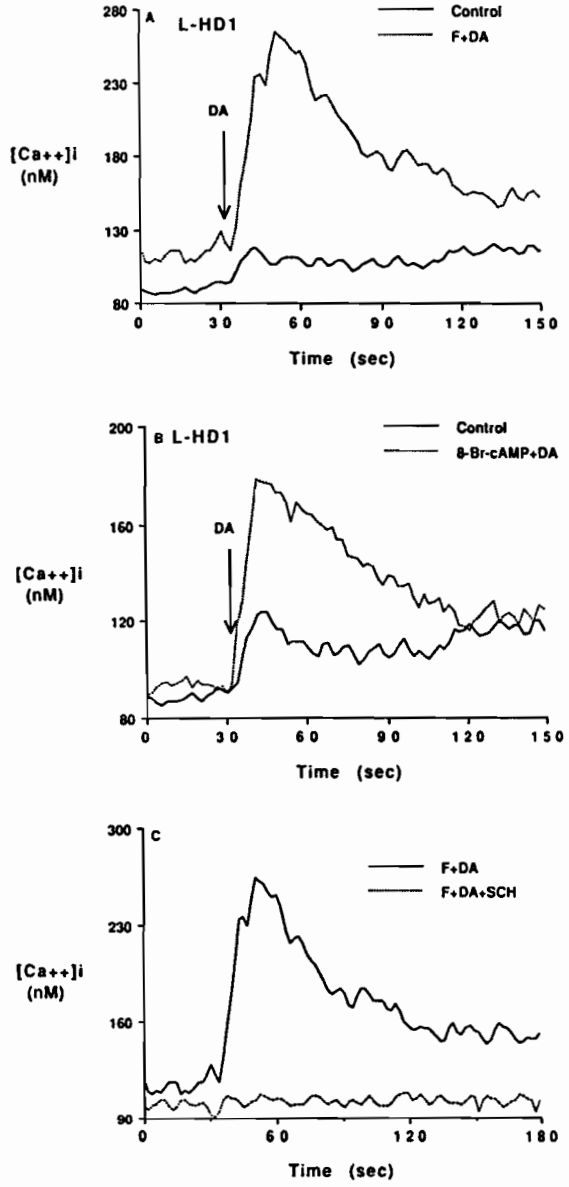

Fig. 5. Preactivation of cAMP-Dependent Protein Kinase Enhances the Dopamine-Induced Increase in $[Ca^{++}]_i$ in L-hD1 Cells

Curves represent a typical experiment which has been repeated at least three times. A, Forskolin pretreatment potentiates the dopamine-induced increase in $[Ca^{++}]_i$. Forskolin (10 μM) was added to the growth medium 3 h before assay. Dopamine (1 μM) was added as indicated. B, 8-Bromo-cAMP enhances the action of dopamine on $[Ca^{++}]_i$. 8-Bromo-cAMP (5 mM) was added to the growth medium 3 h before assay. C, SCH 23390 abolishes the synergistic effect of forskolin and dopamine on $[Ca^{++}]_i$ in L-hD1 cells. The L-hD1 cells were pretreated with 10 μM forskolin for 3 h; 1 μM SCH 23390 was added to the cuvette 1 min before recording; 1 μM dopamine was added as indicated.

to 3-fold over basal level. The enhanced dopamine-induced calcium response is similar in magnitude and time course to response induced by ATP (Fig. 5B) or thrombin (not shown), and thus represents an important transduction pathway in L-hD1 cells. The synergistic effect of cAMP was completely abolished by SCH 23390 (Fig. 5C), indicating mediation of the enhanced calcium signal by activation of the dopamine-D1 receptor.

Dopamine-Enhanced Total Inositol Phosphate (IP) Production in L-hD1 Cells

Activation of PLC and subsequent increase in IP_3 production is the most well known pathway of receptor-mediated mobilization of intracellular calcium pools (1, 26). In L-hD1 cells preequilibrated with [^{3}H]-inositol, 1 μM dopamine increased total IP production by 32 ± 6% (n = 5, Fig. 6A). The action of dopamine was abolished by SCH 23390 (1 μM) and by pretreatment with CTX but not PTX (Fig. 6A). When L-hD1 cells were pretreated with forskolin (10 μM) or 8-bromo-cAMP for 1 h, the maximal response obtained for the dopamine-induced increase of IP production was significantly enhanced by 40% ($P < 0.05$, n = 4, Fig. 6A), similar to potentiation by cAMP of the receptor-mediated release of intracellular calcium stores (see above). These results suggest that the enhancement of the D1 receptor-

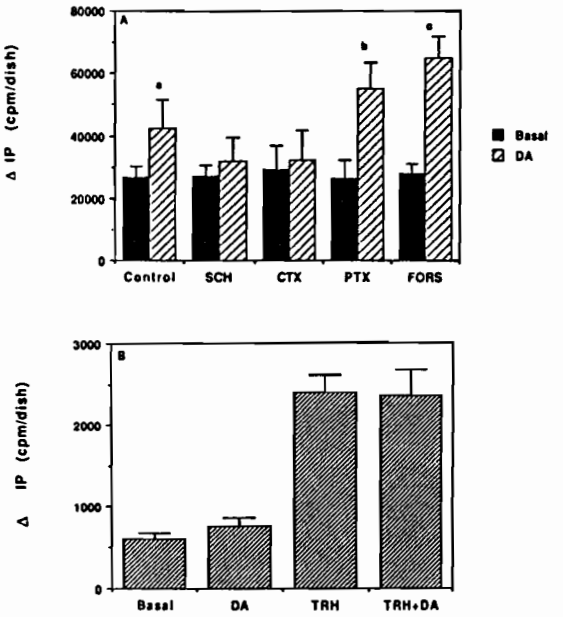

Fig. 6. Activation of the Dopamine-D1 Receptor Enhances Total IP Production in L-hD1 But Not in GH4-hD1 Cells

A, Effects of various compounds on dopamine-induced increase of total IP production. [^{3}H]IP, IP_2, and IP_3 production was measured as described in *Materials and Methods*. a, $P >$ 0.05 compared to basal level, *i.e.* an insignificant change; b, $P < 0.05$ compared to basal level, *i.e.* a significant change; c, $P < 0.05$ compared to the DA-enhanced IP level. Forskolin (10 μM) or 8-bromo-cAMP (5 mM) was added to the medium 3 h before assay. CTX (2 μg/ml) and PTX (10 ng/ml) were added to the growth medium 24 h before assay. Dopamine (DA), 1 μM; SCH 23390 (SCH), 1 μM. B, Dopamine does not alter the total IP production in GH4-hD1 cells. TRH (100 nM) was used.

mediated calcium signal by elevation of cAMP is due to increased IP production. By contrast, in GH4-hD1 cells, dopamine altered neither basal nor TRH-induced increase of total IP production (Fig. 6B). Hence we conclude that in L fibroblast cells, but not in GH pituitary cells, the D1-receptor induces a CTX-sensitive enhancement of PI turnover, which can be potentiated by prior increase in cAMP levels.

DISCUSSION

In this report, we demonstrate that the cloned human dopamine-D1 receptor induces multiple, cell-specific signal transduction pathways. In both GH4-hD1 and L-hD1 cells, the dopamine-D1 receptor elevated cAMP levels, indicating that the receptor stimulates AC activity via coupling to Gs, as expected from previous studies (8–10). In electrically excitable GH4-hD1 cells, the D1 receptor stimulation led to activation of AC and opening of L-type voltage-dependent calcium channels, while in L-hD1 cells stimulation of the same receptor resulted in enhancement of IP production, in addition to stimulation of AC. Each of these signals was blocked by the D1 antagonist SCH 23390 but not by dopamine-D2 receptor antagonists, consistent with the pharmacology of the receptor.

Calcium Signal in GH Cells

Opening of L-type voltage-dependent calcium channels is induced by several Gs-coupled receptors, for example, β-adrenergic receptor in heart (27) and the CRH receptor in AtT-20 cells (28, 29). Studies using bacterially expressed αs *in vitro* have demonstrated that αs alone can activate two effectors, AC and voltage-dependent calcium channels (30). There is evidence that Gs directly activates voltage-dependent calcium channels in cardiac myocytes (27, 30) and indirectly enhances opening of voltage-dependent calcium channels via cAMP-dependent activation of protein kinase A in cardiac myocytes (31), neurons (32, 33), and endocrine cells (28, 29, 34, 35). In the case of the D1 receptor transfected in GH4C1 pituitary cells, the increased influx of calcium via opening of L-type voltage-dependent calcium channels was mediated indirectly by activation of cAMP-dependent protein kinase, since both forskolin and 8-bromo-cAMP mimicked the action of dopamine and did not have an additive effect. We found that preactivation of the dopamine-D1 receptor in GH cells shifted the concentration dependence curve of (±)-Bay K8644 to the left, increasing its potency by 10-fold without changing the maximal response. These results suggest that the dopamine-induced opening of L-type voltage-dependent calcium channels results from a cAMP-dependent modulation of the basal excitability of the channels, possibly by decreasing the threshold for opening of these channels.

A number of approaches have shown that cAMP-dependent stimulation of hormone secretion is mediated indirectly via activation of calcium channels, and that inhibition of channel opening blocks secretion induced by elevation of cAMP (28, 29, 34–36). In GH4C1 cells, enhancement of PRL secretion by vasoactive intestinal peptide, forskolin, and cAMP analogs, which induce a small increase in $[Ca^{++}]_i$ similar to the dopamine-induced effect, is mediated by activation of nifedipine-sensitive calcium channels (34–36). Hence, the small increase in $[Ca^{++}]_i$ induced by elevation of cAMP by dopamine in GH4-hD1 cells is expected to play a crucial role in stimulus-secretion coupling.

Calcium Signal in L Cells

In L-hD1 cells, activation of the D1 receptor induced a different type of increase in $[Ca^{++}]_i$, which was rapid and transient rather than slowly developing and sustained, as in GH cells. In contrast to GH cells we found no evidence for the presence or activation of calcium channels in these cells, in agreement with molecular analyses of these cells (37). The D1-induced calcium increase was mediated by mobilization of ionomycin-sensitive calcium pools (24) and was correlated with an increase in IP accumulation. These observations are consistent with coupling of the D1 receptor to PLC, leading to increased IP_3 levels, and IP_3-mediated release of calcium from intracellular stores (26). While Gs-coupled receptors have not been thought to couple to activation of PLC, recent evidence indicates that for the cloned TSH (38), LH (39), and PTH (40) receptors, such coupling occurs. However, not all Gs-coupled receptors enhance PI turnover and calcium release with the same efficiency. Thus, in L cells, the transfected LH receptor (1.1 pmol/mg protein) caused a 2-fold increase in $[Ca^{++}]_i$, while the transfected vasopressin-V2 receptor (7–8 pmol/mg) and the endogenous prostaglandin E1 receptor had little or no effect on $[Ca^{++}]_i$ (39). The dopamine-D1 receptor (1.6 pmol/mg) displayed intermediate coupling, eliciting a 1.5-fold increase in $[Ca^{++}]_i$. Interestingly, no common structural motif has been identified between receptors which couple efficiently to the PI pathway.

While these data illustrate the receptor specificity of the calcium pathway, the physiological relevance of the pathway must be considered. In the case of the LH receptor, the levels of receptor in ovarian tissue (0.7 pmol/mg) are close to levels expressed in the L cells, and a similar PI-linked response is found in ovarian tissue (39). In striatal membranes, dopamine D1 receptor density has been estimated at 0.4 pmol/mg (10). However, the actual density of receptors in individual cells may be much higher, since brain structures are heterogeneous, hence a small proportion of the membranes is expected to contain dopamine-D1 receptors. Thus, the level of D1 receptor expressed in the L-hD1 cells is high but probably in the physiological range. In this regard, the observation of D1 receptor-induced PI turnover in rat brain slices (13), as well as in retinal horizontal cells (11) and renal cortical membranes (12)

indicates that coupling of a D1-like receptor to PI-linked pathway does occur *in vivo*. What remains unclear is whether the PI response *in vivo* is mediated by the dopamine-D1 receptor or some related receptor subtype yet to be identified (14). Our results indicate that the cloned human dopamine-D1 receptor can activate the PI-linked pathway in L-hD1 cells, and that this pathway is markedly potentiated by prior elevation of cAMP levels.

Sensitivity to CTX

In order to characterize further the pathway by which the dopamine-D1 receptor enhances IP formation in L cells, we tested the actions of CTX on this pathway. First, we ascertained that the dopamine-induced calcium signal was not mediated by cAMP, since it was not mimicked or blocked by forskolin (which elevated cAMP) or 8-bromo-cAMP (which mimics cAMP), which did not alter $[Ca^{++}]_i$ in these cells. In fact, these agents markedly potentiated the dopamine-D1-induced calcium signal (discussed below). In contrast, CTX, which elevates cAMP via ADP-ribosylation and consequent activation of Gs (18), completely blocked the dopamine-induced increase in IP formation and $[Ca^{++}]_i$. Together, these results indicate that dopamine action is blocked directly by CTX, rather than indirectly via increase in cAMP. In addition to blocking dopamine-induced PI turnover in L cells, CTX also prevented the enhancement of cAMP in L and GH cells and calcium influx in GH cells. These results indicate that all dopamine D1 receptor-mediated signals are transduced by CTX-sensitive pathways, probably involving the CTX substrate Gs or a Gs-like protein. In clonal human pituitary cells (41), smooth muscle cells (42), and renal glomerulosa cells (43), pretreatment with CTX also blocks receptor-induced PI turnover, and Gs or a related CTX substrate has been suggested to mediate these actions, although no biochemical evidence for a new CTX substrate has been presented. Thus, we suggest that Gs mediates the two divergent signaling pathways of the cloned D1 receptor in L cells: increase in cAMP accumulation and stimulation of PI turnover and calcium mobilization. While the α-subunit of Gs has been clearly demonstrated to enhance AC activity, there is no evidence that it can activate PLC. It may be that Gs increases PLC activity via release of specific $\beta\gamma$ subunits after agonist binding to certain receptors (*e.g.* LH or dopamine-D1) in light of recent evidence that $\beta\gamma$ subunits can play direct roles in signal transduction (*e.g.* Ref. 44).

Potentiation of the Calcium Signal in L Cells

The action of pretreatment with forskolin or 8-bromo-cAMP to enhance both the dopamine-induced PI turnover and calcium mobilization in L cells demonstrates a potentiation by protein kinase A (PKA) that involves enhanced coupling of the D1 receptor to PLC, rather than a downstream action such as potentiation of IP_3-induced calcium release (45). The target of PKA may be the D1 receptor, Gs, or PLC, but the net effect is enhanced coupling of the receptor to PLC. This potentiation of the calcium signal is opposite to the view that activation of PKA mediates homologous desensitization of Gs-coupled receptors (46–48). In the case of the β-adrenergic receptor, receptor activation causes a Gs-mediated increase in cAMP levels, activating PKA. PKA-induced phosphorylation of the receptor causes uncoupling of the receptor from Gs, decreasing activation of AC (46–48). Similar homologous desensitization has also been reported for the dopamine-D1 receptor (49), yet we observe a sensitization of PLC activation. Thus, rather than simply turning all signaling pathways of a target receptor off or on, kinase activation may perform a more interesting function: to selectively down-regulate certain pathways while up-regulating other pathways, altering the signals generated by the receptor. Since we have observed previously that in transfected L cells, activation of PKC selectively blocks the PI-linked calcium signaling pathway, but not inhibition of cAMP accumulation mediated by the 5-HT1A (6) or dopamine-D2 receptors (50), the pathway-specific alteration of the receptor signaling by protein kinases may be a general phenomenon common to the other G-protein coupled receptors.

MATERIALS AND METHODS

Materials

8-Bromo-cAMP, forskolin, dopamine, CTX, PTX, and 3-isobutyl-1-methylxanthine were purchased from Sigma (St. Louis, MO); [^{3}H]*myo*-inositol (18 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Nifedipine, (±)-Bay K8644, SCH 23390, sulpiride, and spiperone were from Research Biochemical Inc. (Natick, MA). Fura 2-AM was from Molecular Probes (Eugene, OR).

Cell Culture

All cells were grown in monolayer culture on tissue culture dishes (Falcon, Oxnard, CA) at 37 C in a humidified atmosphere with 5% carbon dioxide. Ltk– and L-hD1 were grown in α-minimum essential medium supplemented with 5% fetal bovine serum (GIBCO, Grand Island, NY); GH4C1 and GH4-hD1 cells were grown in F-10 medium with 8% fetal bovine serum. Media were changed 12–24 h before experimentation.

Transfection and Receptor Binding Assay

The 3-kilobase *Eco*RI–*Sac*I gene fragment containing the entire human dopamine-D1 receptor gene (15) was inserted into pZem3 (Promega, Madison, WI). The resulting recombinant was cleaved with *Eco*RI and *Bam*HI, ligated into one of the *Bgl*II sites of the pZem3 vector (15), annealed to an *Eco*RI–*Bam*HI adapter, and annealed to the other *Bgl*II site of the pZem3 vector. This construct was stably cotransfected with pRSVneo into GH4C1 and Ltk– cells as described (6, 15). Cell membranes were prepared by lysis in ice-cold hypoosmotic buffer (15 mM Tris-HCl, 2.5 mM $MgCl_2$, and 1 mM EDTA), homogenization with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at setting 6 for 10 sec, and centrifugation at 800 × *g* for 10 min. The supernatant was saved

and the above steps were repeated once with the remained pellet. All supernatants were centrifuged at 100,000 × *g* for 45 min at 4 C, and the pellets were resuspended in the binding buffer (25 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, and 1 mM EDTA) and stored in −70 C. The binding assay was performed as described previously (15).

Measurement of $[Ca^{++}]_i$

Measurement of $[Ca^{++}]_i$ was performed as described previously (6). In brief, the cells were grown in 100-mm dishes and harvested by trypsinization for Ltk− cells and using calcium-free assay buffer (HBBS: 118.0 mM NaCl, 4.6 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10.0 mM D-glucose, and 20.0 mM 4-(3-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2) containing 5 mM EDTA for GH cells. After centrifugation, the cells were incubated with 2 μM fura 2-AM in HBBS for 30 min at 37 C. They were washed three times in HBBS buffer and finally resuspended in 2 ml HBBS and placed a fluorescence cuvette. Change in fluorescence ratio was recorded on a Perkin Elmer (Norwalk, CT) LS-50 spectrofluorometer and analyzed by computer, based on a dissociation constant of 227 nM for the fura 2-Ca^{++} complex. Calibration of Rmax was performed by addition of 0.1% of Triton X-100 and 20 mM Tris base, and of Rmin by addition of 10 mM EGTA (6, 20). All drugs were added directly to the cuvette from 200-fold concentrated solutions at the time indicated in the figures. In most figures, curves from different samples of the same batch of cells are overlaid. Note that sample-to-sample variation in basal $[Ca^{++}]_i$ (5–10%) is much greater than the intrasample variation, and that differences in basal $[Ca^{++}]_i$ of overlaid data are not significant unless indicated.

cAMP Assay

As we described previously (6), the cells were plated in six-well 35-mm dishes, and media were changed 12–24 h before experimentation. After removal of the medium, cells were preincubated in 2 ml/well HBBS for 5–10 min at 37 C, and the buffer was replaced by 1 ml HBBS containing 100 μM 3-isobutyl-1-methylxanthine and incubated for another 5 min. Various test drugs were added, and incubation continued for 20 min. The buffer was collected for assay using a specific cAMP RIA (ICN, Costa Mesa, CA), as described before (6).

Assessment of PI Turnover

As described previously (6), both L-hD1 and GH4-hD1 cells were grown in six-well 35-mm dishes and were equilibrated for 48 h in regular medium supplemented with 3 μCi/ml or 5 μCi/ml [^{3}H]*myo*-inositol for L and GH cells, respectively. After washing three times with HBBS, the cells were incubated in 2 ml HBBS containing 10 mM LiCl for 2 h, and then the buffer was replaced by 1 ml of the same buffer supplemented with various drugs and incubated for 15 min. The reaction was terminated by aspiration followed by addition of 0.3 ml ice-cold 10% trichloroacetic acid. The lysates were collected and centrifuged at 10,000 × *g* for 15 min. The pellets were disposed of, and the supernatant was extracted three times with 3 ml dimethyl ether. Radioactivity was counted for 5 min in a scintillation counter.

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CHAPTER V DIFFERENTIAL SENSITIVITY OF SHORT AND LONG HUMAN DOPAMINE D2 RECEPTOR SUBTYPES TO PROTEIN KINASE C

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Differential Sensitivity of the Short and Long Human Dopamine D_2 Receptor Subtypes to Protein Kinase C

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Abstract: The human dopamine D_{2L} (long form) and D_{2S} (short form) receptors were expressed separately in mouse Ltk^- fibroblast cells to investigate whether there is a difference in transmembrane signaling of these D_2 receptors. Both receptors induced two signals, a phosphatidylinositol-linked mobilization of intracellular calcium and an inhibition of cyclic adenosine 3'-5' monophosphate (cAMP) accumulation, each with similar response magnitudes and identical pharmacology. Both calcium and cAMP signals were sensitive to pretreatment with pertussis toxin (PTX), indicating mediation by coupling to G_i/G_o proteins. However, the two forms of D_2 receptor were distinguished by acute prior activation of protein kinase C (PKC) with 12-*O*-tetradecanoyl 4β-phorbol 13-acetate (TPA): TPA blocked the D_{2S}-mediated increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$) in a concentration-dependent manner (between 10 n*M* and 1 μ*M*), whereas the D_{2L} receptor-induced increase in $[Ca^{2+}]_i$ was resistant to TPA and was only partially (60%) inhibited by 100 μ*M* TPA. By contrast, TPA did not alter the inhibition of cAMP accumulation induced by activation of either D_{2S} or D_{2L} receptors. We conclude that, in the L cell system, prior activation of PKC differentially modulates the transmembrane signaling of the D_{2L} and D_{2S} receptors, preferentially inhibiting the D_{2S} receptor-mediated calcium signal but not altering the dopamine-induced inhibitory cAMP signal of either receptor subtype.
Key Words: Transmembrane signaling—D_2 receptor subtypes—Cyclic AMP—Calcium—Protein kinase C. **Liu Y. F. et al.** Differential sensitivity of the short and long human dopamine D_2 receptor subtypes to protein kinase C. *J. Neurochem.* **59**, 2311–2317 (1992).

The dopamine D_2 receptor was originally cloned as a cDNA encoding a 415-amino acid receptor, with extensive homology to other G protein-linked receptors, particularly in the seven putative transmembrane domains (Bunzow et al., 1988). Unlike other members of this gene family (Dohlman et al., 1991), the dopamine D_2 receptor gene gives rise to two forms of the receptor arising from the alternative splicing of the sixth exon, which encodes a 29-amino acid domain (Giros et al., 1989; Grandy et al., 1989; Monsma et al., 1989). The 29-amino acid insert is located in the third cytoplasmic loop of the D_{2L} receptor, a region that contains potential phosphorylation sites (Civelli et al., 1991) that could be involved in receptor desensitization (Bouvier et al., 1989; Hausdorff et al., 1989). Furthermore, portions of the third cytoplasmic loop that are adjacent to the putative membrane-spanning domains play an important role in coupling to G proteins and generation of receptor-mediated responses (Strader et al., 1987; Kobilka et al., 1988; O'Dowd et al., 1988; Liggett et al., 1991). Because the 29-amino acid insert is located close to the fifth transmembrane domain, a potential role in altering G protein coupling has been sought. To date, however, no clear-cut difference in receptor pharmacology or function of the short and long forms of the D_2 receptor has been documented, although small differences in potency have been claimed (Montmayeur and Borrelli, 1991).

The present work was undertaken to investigate further whether there is a difference in transmembrane signaling between these two dopamine receptor subtypes. The human dopamine D_{2L} and D_{2S} receptors were expressed in Ltk^- fibroblast cells. When these receptors were expressed in receptor-negative Ltk^- fibroblast cells, we found that both human D_{2L} and D_{2S}

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Abbreviations used: $[Ca^{2+}]_i$, cytosolic free calcium concentration; cAMP, cyclic adenosine 3',5'-monophosphate; PKC, protein kinase C; PTX, pertussis toxin; TPA, 12-*O*-tetradecanoyl 4β-phorbol 13-acetate.

receptors increased the cytosolic free calcium concentration ($[Ca^{2+}]_i$) and inhibited forskolin-induced activation of adenylyl cyclase with a similar pharmacological profile. However, the two receptor forms were differentially modulated by protein kinase C (PKC). Our results suggest that receptor cross-talk via activation of PKC may selectively uncouple the calcium signaling of the dopamine D_{2S} receptor.

MATERIALS AND METHODS

Materials

Sera, media, and G418 (Geneticin) were purchased from GIBCO (Grand Island, NY, U.S.A.). Dopaminergic ligands were from Research Biochemicals Inc. (Natick, MA, U.S.A.). 12-*O*-Tetradecanoyl 4β-phorbol 13-acetate (TPA) and thrombin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Methods

Murine Ltk$^-$ cells were transfected with either the *hD2L* cDNA or the *hD2S* cDNA, as described previously (Grandy et al., 1989). Scatchard analysis of specific [^{3}H]spiperone binding in membranes prepared from hD2L cells yielded a B_{max} value of 161 ± 2 fmol/mg of protein; membranes from hD2S cells had a B_{max} of 153 ± 18 fmol/mg of protein. Membranes from nontransfected Ltk$^-$ cells had no specific binding for [^{3}H]spiperone (Bunzow et al., 1988). $[Ca^{2+}]_i$ was measured as described previously (Liu and Albert, 1991). The cells were preloaded with 2 μM fura 2-AM for 30 min at 37°C and washed three times with Hanks' balanced salt solution (118 mM NaCl, 4.6 mM KCl, 1.0 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, and 20 mM HEPES, pH 7.2) before being placed in a fluorescence cuvette. Any change in fluorescence intensity was recorded on a Perkin-Elmer model LS-50 spectrofluorometer and analyzed by computer, based on a K_D of 227 nM for the fura 2–Ca^{2+} complex.

RESULTS

Dopamine-induced increase in $[Ca^{2+}]_i$ in LhD2L and LhD2S cells

Change of $[Ca^{2+}]_i$ was measured using an intracellular-trapped fluorescent indicator, fura 2-AM (Liu and Albert, 1991). Dopamine (100 nM) evoked nearly identical increases in $[Ca^{2+}]_i$ in LhD2L and LhD2S cells (Fig. 1A and B). The maximal increase in $[Ca^{2+}]_i$ was 3.2 ± 0.2-fold (n = 6) above basal values for LhD2L cells and 3.0 ± 0.3-fold (n = 6) for LhD2S cells. This response was not observed in nontrans-

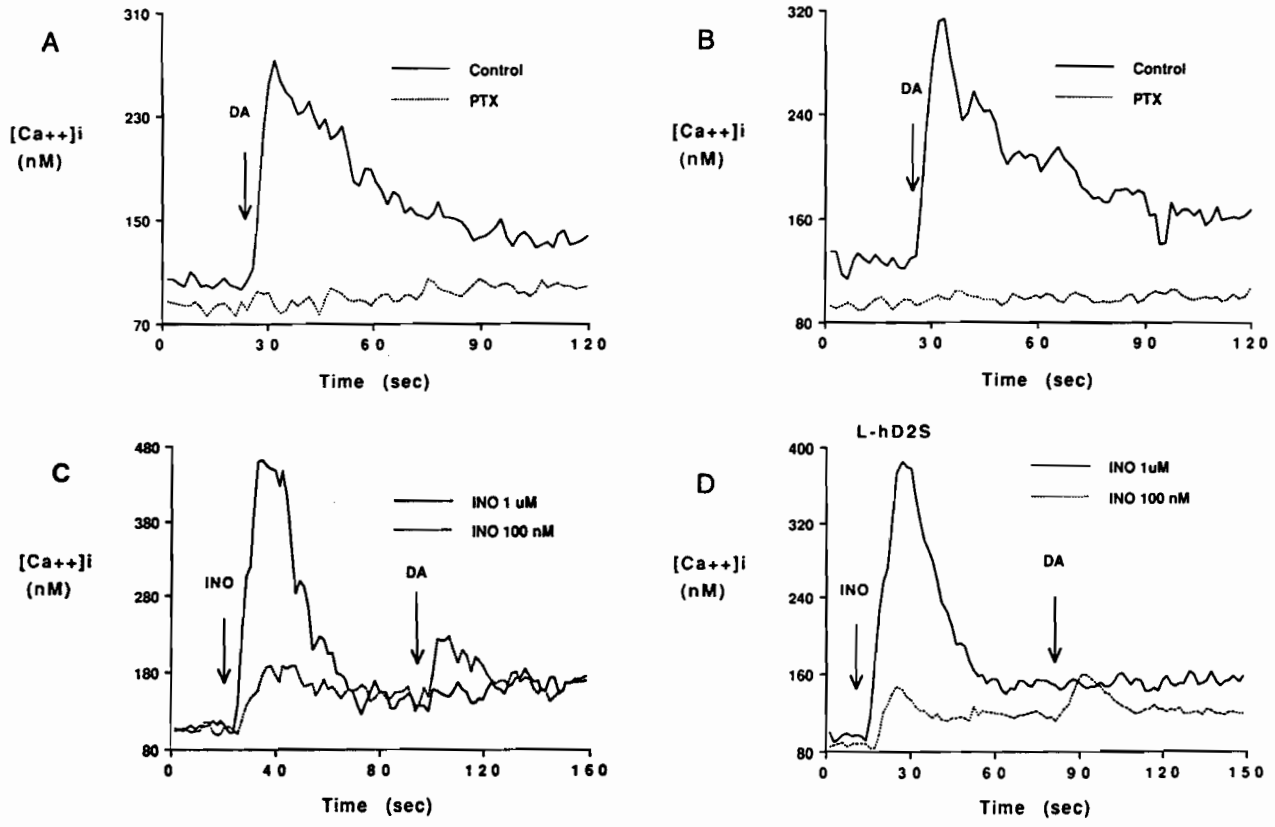

FIG. 1. Dopamine (DA) increases in $[Ca^{2+}]_i$ in LhD2L and LhD2S cells. All curves represent one of at least four independent experiments performed in duplicate measuring $[Ca^{2+}]_i$ in cells loaded with fura 2. **A and B:** DA increases in $[Ca^{2+}]_i$ in LhD2S (A) and LhD2S (B) cells. PTX (10 ng/ml) added to the growth medium 16 h before experimentation completely abolishes these responses. DA (100 nM) was added to the cuvette as indicated. **C and D:** Ionomycin (INO) blocks the DA-induced increase in $[Ca^{2+}]_i$ in LhD2L (C) and LhD2S cells (D). DA and INO were both used at 100 nM.

fected Ltk⁻ cells or in Ltk⁻ cells expressing transfected rat serotonin 5-HT_{1A} receptors (data not shown). Pretreatment with pertussis toxin (PTX; 10 ng/ml, 16 h) completely abolished the action of dopamine in both cell lines (Fig. 1A and B), suggesting mediation by PTX-sensitive G proteins.

Ionomycin, an ionophore that induces release of calcium mainly from intracellular calcium stores (Albert and Tashjian, 1986), was used to induce release of calcium pools before stimulation with dopamine. Figure 1C shows that addition of ionomycin partially (100 n*M*) or completely (at 1 μM) blocked the action of dopamine in LhD2L cells, depending on the extent of calcium mobilization induced by ionomycin. Similar results were obtained using LhD2S cells (Fig. 1D). These results demonstrate that the dopamine-induced increase in $[Ca^{2+}]_i$ in LhD2L and LhD2S cells is mainly due to ionomycin-sensitive mobilization of intracellular calcium stores.

Increases in $[Ca^{2+}]_i$ induced by dopamine were concentration dependent (Fig. 2A and B), with an EC_{50} of 10 ± 2 and 25 ± 4 n*M* for LhD2S and LhD2L cells, respectively (Fig. 2C). In the presence of 10 μM sulpiride, a dopamine D_2 receptor antagonist, the actions of dopamine in LhD2L and LhD2S cells were completely abolished (data not shown). These results indicated that the dopamine D_{2L} and D_{2S} receptors mediated increases in $[Ca^{2+}]_i$ in L cells with a similar pharmacological profile, consistent with their similarity in binding properties.

Activation of PKC by TPA inhibits the action of dopamine

To examine the role of PKC activity in regulating dopamine D_2 receptor function, LhD2L and LhD2S cells were pretreated with TPA 5 min before addition of 100 n*M* dopamine (Fig. 3). LhD2L and LhD2S cells differed in sensitivity to acute pretreatment with TPA: At a concentration of 1 μM, TPA completely abolished the action of dopamine in LhD2S cells (Fig. 3A), but it only slightly attenuated (<40%) the dopamine response in LhD2L cells (Fig. 3B). These results were consistently observed in six independent experiments in which change in $[Ca^{2+}]_i$ was measured in parallel in LhD2L and LhD2S cells. In LhD2L cells (Fig. 3C) the maximal inhibition by 100 μM TPA of the dopamine response was incomplete (60% inhibition, n = 4), further evidence of the greater resistance of the D_{2L} receptor subtype to PKC activation. The TPA-induced inhibition of the dopamine responses was concentration dependent but also depended on the dopamine concentration used. At 100 n*M* dopamine, the IC_{50} value for TPA action was ~100 n*M* in LhD2S cells and >1 μM for LhD2L cells (Fig. 3D). These results indicate that acute activation of PKC preferentially inactivates the D_{2S}-induced calcium signal in L cells and incompletely attenuates the D_{2L} response with a 10-fold lower potency. By contrast, elevation of cyclic adenosine 3',5'-monophosphate

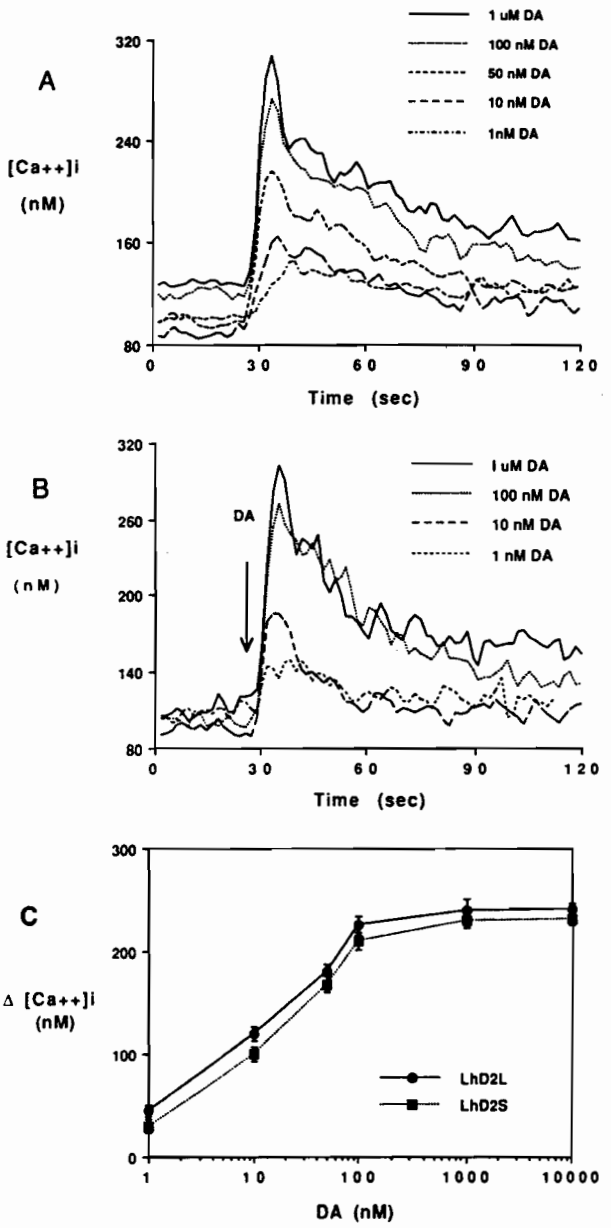

FIG. 2. Pharmacological profile of the dopamine (DA)-induced increase in $[Ca^{2+}]_i$ in LhD2L and LhD2S cells. **A and B:** Concentration dependence of the DA-induced increase in $[Ca^{2+}]_i$ in LhD2L (A) and LhD2S (B) cells. Various concentrations of DA were added to the cuvette as indicated. **C:** Concentration dependency of the DA-induced increase in $[Ca^{2+}]_i$ in LhD2L and LhD2S cells. Data are plotted as peak of $[Ca^{2+}]_i$ increase − basal $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) versus DA concentrations and are mean ± SD (bars) values of three independent experiments.

(cAMP) levels using acute (5-min) pretreatment with 10 μM forskolin did not alter basal or dopamine-induced increase in $[Ca^{2+}]_i$ (data not shown), indicating that protein kinase A does not inhibit this pathway as does PKC.

To ascertain whether the differential sensitivities of D_{2L} and D_{2S} receptors to TPA pretreatment were due to clonal variation in TPA responsiveness between

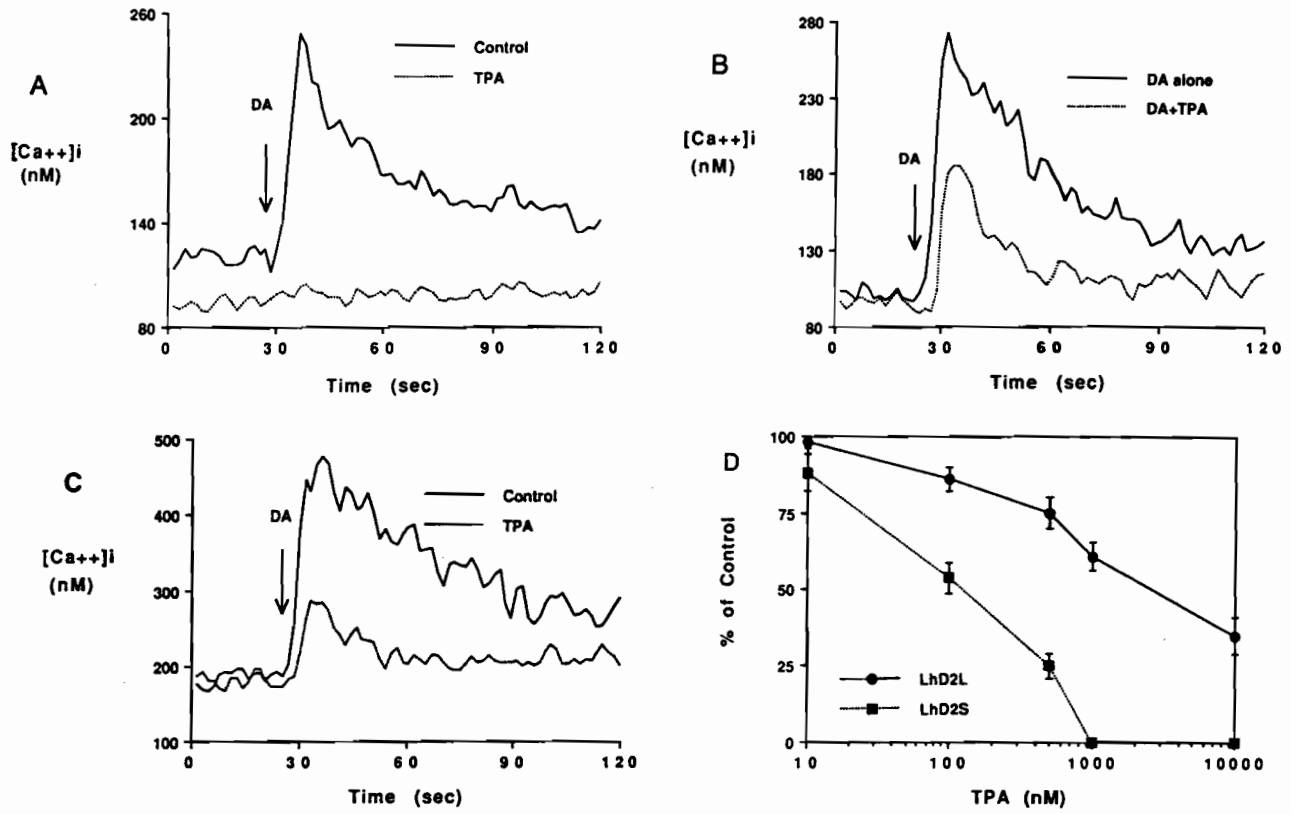

FIG. 3. TPA inhibits the dopamine (DA)-induced increase in $[Ca^{2+}]_i$ in LhD2L and LhD2S cells. All curves were from a typical experiment, which was repeated six times with similar results. The cells were incubated with 1 μM TPA for 5 min before recording. DA (100 nM) was added as indicated. **A:** TPA (1 μM) completely abolishes the DA induced-increase in $[Ca^{2+}]_i$ in LhD2S cells. **B:** TPA (1 μM) partially inhibits the DA-induced increase in $[Ca^{2+}]_i$ in LhD2L cells. **C:** LhD2L cells were incubated as above with 100 μM TPA and treated with 100 nM DA as indicated. **D:** Concentration dependency of TPA-induced inhibition of the maximal increase in $[Ca^{2+}]_i$ in LhD2L and LhD2S cells. The error bars are the SD values calculated from six independent experiments performed in duplicate and in parallel with D_{2S} and D_{2L} cells.

the LhD2L and LhD2S cells, the sensitivity of the endogenous thrombin-induced calcium mobilization to TPA was measured in both clones (Table 1). Thrombin (0.2 U/ml) induced an increase in $[Ca^{2+}]_i$ that was identical in both cell lines, with a peak of 3.5-fold basal $[Ca^{2+}]_i$. In both cell lines, TPA inhibited the thrombin-induced response to a similar extent, by 45% at 10 nM TPA and completely at 100 nM TPA, indicating that the potency of TPA toward this response was identical in both clones. As further evidence of the selective inactivation of the D_{2S} receptor by TPA, we found that TPA also completely inhibited the calcium response of the rat D_{2S} receptor expressed in L cells [RGB-2 cells (see Vallar et al., 1990)]. The IC_{50} value for TPA action on the rat D_{2S} receptor was 80 nM (data not shown), similar to that obtained for the human D_{2S} receptor (Fig. 3D). Thus, differential sensitivity of the D_2 receptor subtypes appears to be a property of the receptors and not due to clonal variation.

TPA does not alter dopamine-induced inhibition of cAMP accumulation

Dopamine D_2 receptors inhibit adenylyl cyclase via coupling to PTX-sensitive G proteins in many systems (Enjalbert and Bockaert, 1983; Enjalbert et al., 1988; Albert et al., 1990). In both LhD2L and LhD2S cells, 100 nM dopamine did not alter the basal cAMP accumulation but decreased the forskolin-induced enhancement of cAMP accumulation by 90%. The actions of dopamine were concentration dependent,

TABLE 1. *Inhibition of thrombin-induced calcium increase by TPA in LhD2L and LhD2S cells*

	Thrombin-induced increase in $[Ca^{2+}]_i$ (-fold basal)	
Pretreatment	LD2L	LD2S
No TPA	3.4 ± 0.20 (n = 6)	3.5 ± 0.25 (n = 4)
TPA		
10 nM	2.4 ± 0.12 (n = 3)	2.35 ± 0.15 (n = 3)
100 nM	1.0 (n = 2)	1.0 (n = 2)

The thrombin (0.2 U/ml)-induced increase in $[Ca^{2+}]_i$ in LhD2L cells and LhD2S cells is expressed as fold basal $[Ca^{2+}]_i$ to normalize the data; thus, a value of 1.0 indicates no stimulation over basal $[Ca^{2+}]_i$. The indicated concentrations of TPA were added 5 min before addition of thrombin, and the peak of calcium concentration increase was measured. Data are mean ± SE values.

with an IC_{50} of 18 ± 1.4 n*M* (n = 3) for LhD2L cells and 12 ± 0.8 n*M* (n = 3) for LhD2S cells (data not shown). Sulpiride (10 μM) or pretreatment for 16 h with PTX (10 ng/ml) completely abolished the actions of dopamine (Fig. 4). These results indicate that both the dopamine D_{2L} and D_{2S} receptors are linked to inhibition of adenylyl cyclase via coupling to G_i/G_o proteins in L cells with a similar pharmacological profile. Acute pretreatment with 1 μM TPA did not change basal or the forskolin-induced increase in cAMP accumulation and did not block the inhibition action of dopamine in LhD2L or LhD2S cells (Fig. 4). Thus, uncoupling of the dopamine receptor by activation of PKC is not only receptor selective, but is also signal specific: Acute activation of PKC selectively attenuated the stimulatory calcium response pathway, without affecting the inhibitory cAMP pathway of the dopamine D_2 receptor.

DISCUSSION

These studies demonstrate two important aspects of the transmembrane signaling of the dopamine D_2 receptor: (a) The calcium signal induced by the D_{2L} receptor is incompletely inhibited and more resistant to PKC activation than the calcium signal induced by the D_{2S} receptor. (b) Prior activation of PKC selectively inhibits the dopamine D_2 receptor-induced calcium signal without altering the inhibitory cAMP signal.

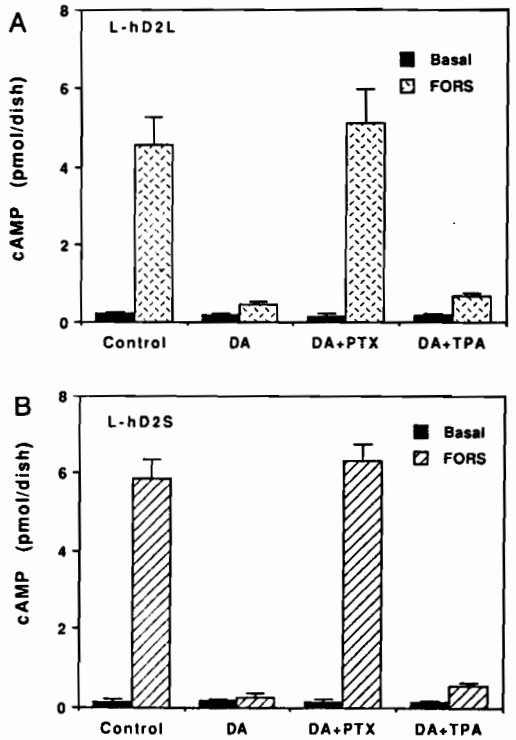

FIG. 4. Effects of various compounds on dopamine (DA)-induced inhibition of cAMP accumulation in (**A**) LhD2L and (**B**) LhD2S cells. The cAMP accumulation was measured as described previously (Albert et al., 1990). Data are mean ± SD (bars) values of three independent experiments performed in triplicate. PTX (10 ng/ml) was added to the growth medium 16 h before experimentation and was not present during assay. Forskolin (FORS), DA, and TPA were used at 10 μM, 100 n*M*, and 10 μM, respectively.

We found no significant difference in the transmembrane signaling of the short and long forms of the D_2 receptors expressed separately in L cells. Thus, the 29-amino acid insert of the long form does not appear to alter directly the signaling phenotype of the D_2 receptor in L cells. The major difference we observed was in the inhibitory modulation of the receptor by PKC, with the D_{2S} form being more sensitive to PKC than the D_{2L} form. Control experiments indicated similar activity of TPA in all clones, and selectivity of PKC appears to be due to a difference at the level of receptor structure. The 29-amino acid insert of the long form does not contain potential PKC phosphorylation sites (Kemp and Pearson, 1990). However, the presence of the 29-amino acid insert creates a potential kinase C pseudosubstrate domain [residues 267–275, RRRXXA*ARR (Kemp and Pearson, 1990)] in the third cytoplasmic loop, which could competitively inhibit the enzyme. There are several potential phosphorylation sites in the second and third cytoplasmic loops of the D_2 receptor near domains thought to be important for receptor interactions with G proteins. Phosphorylation of these residues by PKC may induce uncoupling of the receptor with particular G proteins (Elazer et al., 1989; Bates et al., 1990; Elazar and Fuchs, 1991) and loss of the calcium response. If the presence of the 29-amino acid insert prevents phosphorylation at a crucial residue, the D_{2L} receptor should become resistant to inhibition by PKC.

The in vivo role of the dopamine D_2-induced calcium mobilization is unclear at present. In the striatum (Cooper et al., 1986) and anterior pituitary (Enjalbert and Bockaert, 1983; Enjalbert et al., 1988), dopamine D_2 receptors exhibit an inhibitory signaling phenotype, linked to inhibition of adenylyl cyclase and opening of potassium channels to decrease $[Ca^{2+}]_i$ (Albert et al., 1990; Vallar et al., 1990). However, a subpopulation of dispersed pituitary cells display the stimulatory pathway as evidenced by dopamine D_2 receptor-induced calcium mobilization (Winiger et al., 1987). When expressed in L fibroblast cells, the D_2 receptor behaves as a stimulatory receptor, where enhancement of $[Ca^{2+}]_i$ is the major signal and the dopamine-induced decrease in cAMP levels is an accessory signal, because dopamine does not alter the basal cAMP level. The identification of the PTX-sensitive stimulatory calcium pathway for *hD2*-receptor subtypes expressed in L fibroblasts extends and confirms observations using the rat D_{2S} and serotonin 5-HT_{1A} receptors, which also induce a PTX-sensitive enhancement of $[Ca^{2+}]_i$ in L cells (Vallar et al., 1990; Liu and Albert, 1991). These studies have indicated

that the receptor-induced calcium response is due to enhanced phosphatidylinositol turnover, which increases levels of inositol trisphosphate to induce release of calcium from intracellular stores and enhances diacylglycerol levels to activate PKC (Berridge and Irvine, 1989). The stimulatory calcium signaling pathway of the 5-HT_{1A} receptor is also associated with PTX-sensitive growth-promoting activity and tumorigenic potential in BALB/c-3T3 fibroblasts (Abdel-Baset et al., 1992) and may play a role in cell growth and development.

The present studies exemplify a second concept concerning hormone receptor signaling, namely, pathway-selective heterologous desensitization: PKC selectively inactivated the D_2 receptor-induced increase in $[Ca^{2+}]_i$ but had no effect on inhibition of cAMP accumulation in the same cell system. Another D_{2L}-induced pathway that is not blocked by acute TPA addition is the cAMP-independent potentiation of arachidonic acid release in Chinese hamster ovary cells (Kanterman et al., 1990). These results suggest that activation of PKC serves as a negative feedback pathway to inhibit specifically phosphatidylinositol-linked calcium signaling of the D_2 receptors, without altering the other receptor-induced signals. Similarly, we have previously reported that acute TPA pretreatment selectively inhibits the calcium signal of the rat serotonin 5-HT_{1A} receptor expressed in L cells, without altering the serotonin-induced inhibition of cAMP accumulation (Liu and Albert, 1991). Numerous studies have shown that acute phosphorylation of the β_2-adrenergic receptor leads to receptor uncoupling (Bouvier et al., 1989; Hausdorff et al., 1989; Lefkowitz et al., 1990), but only one signaling pathway of the receptor has been examined, namely, the stimulation of adenylyl cyclase. Our results demonstrate that activation of a protein kinase can selectively alter the transmembrane signaling of G protein-coupled receptors, uncoupling certain pathways yet leaving others functionally intact, as suggested in another system (Northwood and Davis, 1990). It remains to be determined whether such TPA-induced alteration of receptor signaling has a developmental or pathological significance in vivo.

In conclusion, we have shown that although D_{2S} and D_{2L} elicit identical responses when expressed in L fibroblast cells, the D_{2S} receptor is more sensitive to the signal-specific blockade by TPA than the D_{2L} receptor by at least one order of magnitude.

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CHAPTER VI G PROTEIN SPECIFICITY IN RECEPTOR-EFFECTOR COUPLING

G Protein Specificity in Receptor-Effector Coupling

Analysis of the roles of G_o and G_i2 in GH4C1 pituitary cells

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[1]**Abbreviations:** G protein, guanine nucleotide-binding regulatory protein; G_i, G_o, G_s, G_q, G protein subtypes; $[Ca^{2+}]_i$, cytosolic free calcium concentration; PTX, pertussis toxin; 5-HT, 5-hydroxytryptamine, serotonin; VIP, vasoactive intestinal peptide; RT-PCR, reverse transcription-polymerase chain reaction; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine.

In rat pituitary GH4C1 cells, activation of transfected dopamine D_2 receptors (long, D_{2L}, or short, D_{2S}, form) and endogenously expressed somatostatin and muscarinic M4 receptors induced inhibition of cAMP synthesis and of Bay K 8644-induced calcium entry via pertussis toxin-sensitive G proteins. In order to analyze the role of α_o and α_i2 in relaying of these signals, α_o- or α_i2- antisense constructs were separately and stably transfected into GH4C1 cells. RT-PCR and Western blot analysis indicated specific ablation of α_o or α_i2 in the antisense transfectant clones. Elimination of α_o selectively abolished receptor-mediated closure of calcium channels. Notably, the action of dopamine D_{2L} receptor was partially (about 30%) retained. By contrast, depletion of α_i2 selectively impaired receptor-mediated inhibition of cAMP accumulation. Inhibition of basal cAMP synthesis by any of the four receptors studied was blocked in α_i2-depleted clones. Additionally, dopamine D_{2L}, somatostatin and muscarinic M4 receptor-mediated inhibition of vasoactive intestinal peptide-stimulated cAMP formation was also abolished. Remarkably, somatostatin even potentiated (by 30%) the action of vasoactive intestinal peptide in α_i2-antisense clones. In contrast, the action of dopamine D_{2S} receptor on stimulated cAMP synthesis remained largely unaltered. The results demonstrate that α_o specifically triggers receptor-induced closure of calcium channels whereas α_i2 specifically mediates inhibition of adenylyl cyclase in GH4C1 cells. Furthermore, the data suggest that G_i protein specificity in receptor coupling to inhibition of adenylyl cyclase depends critically on the activity state of the enzyme. Moreover, the results indicate an essential difference in coupling of dopamine D_{2L} and D_{2S} receptors to G proteins.

Heterotrimeric G proteins[1], composed of α subunit and ßγ dimer, provide the key connection between numerous receptors and various effectors, such as adenylyl cyclase, calcium and potassium channels, and phospholipases (1-4). Agonist-activated receptors catalyze exchange of GDP to GTP at G protein α subunits, apparently resulting in dissociation of α-GTP from the receptor and ßγ dimer. GTP-liganded α subunit and free ßγ dimer then regulate various effector activities to alter the intracellular levels of second messengers (2-4). The intrinsic GTPase activity of α subunits hydrolyzes GTP to GDP, thereby creating the inactive GDP-ligated heterotrimer.

Characterization of individual G protein-coupled receptor-linked signalling pathways is one of the central issues in receptor research. Given the numerous possible combinations of currently identified α, ß and γ subunits (5), a wide diversity of receptor-G protein signalling pathways could be anticipated. However, the majority of receptors interact with only one set of G protein classes, e.g. G_s, G_i/G_o, or G_q/G_{11} (6-9), although receptors interacting with more than one set of G protein class, e.g. G_s and G_q, have been found. For example, glucagon, parathyroid and luteinizing hormone receptors can mediate both stimulation of adenylyl cyclase via G_s and activation of phospholipase C via G_q (10-12). Generally, PTX-sensitive G protein-coupled receptors can interact with more than one member of this class (3). This has been suggested by reconstitution of purified receptors and G proteins in liposome vesicles (13), by immunoprecipitation of receptors using antibodies specific for individual α_i/α_o subunits (14,15) and by receptor-stimulated labeling of α subunits by cholera toxin-catalyzed ADP-ribosylation (16). Furthermore, microinjection of antisense oligonucleotides to α_o has demonstrated that somatostatin receptors specifically link to α_{oA}, whereas muscarinic M4 receptors specifically

couple to α_{oB} to mediate inhibition of calcium channels in GH3 pituitary cell (17). Thus, one receptor can apparently link to different G proteins to regulate distinct effectors, while coupling of different receptors to the same effector can be accomplished by distinct G proteins. However, the specifity of a single receptor to couple to multiple Gi/Go proteins in intact cells has not been analyzed.

GH4C1 cells express all known three α_i subunits and the two types of α_o as well as various effectors (e.g. adenylyl cyclase, calcium and potassium channels) which are regulated by PTX-sensitive G proteins. Thus, this cell system provides an excellent model to study the specificity of PTX-sensitive G proteins in coupling of receptors to effectors. In order to evaluate the contribution of α_i2 and α_o subunit in coupling of transfected dopamine D_{2L} and D_{2S}, and endogenously expressed muscarinic M4 and somatostatin receptors (18,19) to inhibition of adenylyl cyclase and voltage-dependent calcium channels in GH4C1 pituitary cells, α_o or α_i2 antisense constructs were stably introduced into GH4C1 cells to "knockout" α_i2 or α_o subunits. We report here essential depletion of α_o or α_i2 from GH4C1 cells. Characterization of these α_o or α_i2 deficient antisense clones indicates that α_o specifically triggers closure of calcium channels, while α_i2 links receptors to inhibition of cAMP synthesis, and that the existence of α_i2 is essential for all four receptor-mediated inhibition of unstimulated cAMP synthesis. Moreover, the results suggest differences in interacting with G_o and G_i2 by long and short forms of dopamine D_2 receptors.

EXPERIMENTAL PROCEDURES

Materials. Dopamine, somatostatin, PTX, IBMX and VIP were purchased from Sigma, St. Louis, MO. Hygromycin B was from Calbiochem, La Jolla, CA. (±)Bay K8644 was from

Research Biochemical Inc., Natick, MA. Fura 2-AM was from Molecular Probes, Eugene, OR. Rat G protein α_o and α_i2 subunit cDNAs were gifts of Dr. R. Reed. G protein α and ß antibodies were kindly donated by Dr. D. Manning and Dr. G. Schultz.

Cell Culture. Rat pituitary GH4C1 cells were transfected with human dopamine D_{2L} receptor cDNA, as described previously (20). Scatchard analysis of specific [^{3}H]spiperone binding in membranes prepared from GH4D2L cells yielded a B_{max} value of 248 $\pm$ 10 fmol/mg of protein; B_{max} obtained from membranes prepared from GH4ZR7 cells (transfected with rat dopamine D_{2S} receptors) was about 0.4 pmol/mg of protein (ion-induced cells), as reported previously (21). All cells were grown as monolayer in Ham's F10 medium with 8% fetal bovine serum, at 37°C in a humidified atmosphere, with 5% carbon dioxide. Media were changed 12-24 h prior to experimentation. **Preparations of the antisense clones.** The 2.1 kb α_{oA} *Eco*RI cDNA fragment or 1.8 kb α_i2 *Eco*RI cDNA fragment (22) were ligated into *Eco*RI-cut pcDNA (Invitrogen) in the reverse orientation with respect to the cytomegalovirus promoter, resulting in α_o and α_i2 antisense expression vector, poDNA and pi2DNA, respectively. The constructs were checked by restriction enzyme analysis and by DNA sequencing. A modified transfection procedure was used: 200 μg of poDNA or pi2DNA and 30 μg of hygromycin B resistant plasmid, PY-3, were co-introduced into G418-resistant GH4ZR7 or GH4D2L cells by standard calcium phosphate coprecipitation protocol (23). The first-step selection was initiated after 24h by adding 150 μg/ml of hygromycin B into the culture medium to select the clones with expression of the antisense RNA and to allow the clones to adapt the cytotoxicity of hygromycin B. After three weeks, the concentration of hygromycin B was raised to 250-400 μg/ml to select clones with the highest levels of the antisense RNA. Isolated clones were propagated, total RNA was prepared

from them and screened by RT-PCR analysis to identify positive clones using a pair of oligonucleotides specific for each α subunit (α_o: 5'-CCGAGCGATGCGAGTTCT-3'and 5'-CTATCAAGATTCCAGCGG-3', α_i2: 5'-CACTACCTGTGAGGAAGA-3'and 5'-ACTCCTCCAGACATAGG-3'). Membranes (50 μg/lane) prepared from the positive antisense clones were solubilized, electrophoresed, transferred to nitrocellulose membranes, and probed with specific antibodies as described (24). Densitometries scanning of the blots was done on the Scanmaster 3 densitometer (Howtek, Hudlson, NH). The data were digitalized and quantitated using the Masterscan analysis program (Scanalytric Billerica, MA), and reconstructed as Masterscan images presented in figures. This analysis allows enhanced resolution of weak signals.

Measurement of $[Ca^{2+}]_i$. Measurement of $[Ca^{2+}]_i$ was performed as described previously (8). In brief, cells were harvested by incubation in calcium-free HBBS containing 5 mM EDTA. The cells were washed once with HBBS (118 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 20 mM Hepes, pH 7.2) and then incubated for 30 min at 37°C in the presence of 2 μM Fura-2AM. They were then diluted to 10 ml, centrifuged, washed twice with HBBS buffer, and finally resuspended in 2 ml HBBS and placed in a fluorescence cuvette. Change in fluorescence ratio was recorded on a Perkin Elmer (Buckinghamshire, England) LS-50 spectrofluorometer and analyzed by computer, based on a K_D of 227 nM for the Fura 2-Ca complex. Calibration of R_{MAX} was performed by addition of 0.1% Triton X-100 and 20 mM Tris base, and of R_{MIN} by addition of 10 mM EGTA (8). All experimental compounds were added directly to cuvette from 200-fold concentrated test solutions at times indicated in the figures.

cAMP assay. Measurement of cAMP was performed as described previously (8). In brief, the cells were plated in six-well 35-mm dishes. After removal of the medium, cells were preincubated in 2 ml/well HBBS for 5-10 min at 37°C, the buffer was replaced by 1 ml of HBBS containing 100 μM IBMX, and the incubation was continued for another 5 min. Then, the various test compounds were added to the wells, and the cells were incubated at room temperature for 20 min. The buffer was collected for cAMP assay using a specific radioimmunoassay (ICN) as described before (8).

Miscellaneous. Protein was determined according to Lowry et al. (25). Statistical analysis was performed using Student's t test. A P value of < 0.05 was taken as significant.

RESULTS

Characterization of G protein signalling in GH4ZR7 and GH4D2L cells. The ability of dopamine, somatostatin, and carbachol, each at a maximally effective concentration, to inhibit cAMP accumulation and calcium entry was examined in GH4ZR7 and GH4D2L cells, expressing dopamine D_{2S} and D_{2L} receptors, respectively. In both cell types, dopamine (1μM), somatostatin (0.25 μM), and carbachol (10 μM) induced inhibition of cAMP accumulation in unstimulated cells and in cells stimulated with VIP (0.2 μM)(Fig. 1). The rank order of efficiency was dopamine $>$ somatostatin $\geq$ carbachol (Table 1). Dopamine, somatostatin, and carbachol also inhibited the increase in $[Ca^{2+}]_i$ caused by the agonist of dihydropyridine-sensitive calcium channels, Bay K 8644 (1 μM) (Fig. 2), with the same rank order as obtained from measurement of cAMP levels (Table 1). All four receptor-induced inhibition of both cAMP accumulation and calcium entry were abrogated by pre-treatment of the cells with PTX,

suggesting involvement of G_i/G_o proteins in receptor-effector coupling (data not shown). No difference in the dopamine actions between GH4ZR7 and GH4D2L cells was observed, indicating that the two forms of dopamine D_2 receptors have a similar extent of coupling to these effectors.

GH4C1 cells endogenously express somatostatin and muscarinic M4 receptors, each about 0.2 pmol/mg protein (18,19), similar to the amounts of the transfected dopamine D_{2S} and D_{2L} receptors (about 0.4 and 0.25 pmol/mg protein, respectively). Furthermore, a similar level (1 pmol/mg protein) of transfected 5-HT1A receptors had an even weaker inhibitory activity in GH4C1 cells than somatostatin or muscarinic M4 receptors (8). Hence, the results suggest that the coupling of dopamine D_2 receptors to G proteins is more efficient than that of somatostatin and muscarinic M4 receptors. Indeed, dopamine exhibited greater efficacy on stimulation of high affinity GTPase activity than somatostatin and carbachol (Y.F.Liu, T. Wieland & K.H. Jakobs, unpublished observation). These results suggest a variation in the number of G proteins activated by different receptors, which results in difference in efficacies in coupling to different effectors.

Elimination of α_o and α_i2 subunits from GH4ZR7 and GH4D2L cells. To address the specificity of distinct G proteins in receptor-effector onteraction, we aimed to ablate the gene expression of α_o or α_i2 in GH4C1 cells. Since G protein α_i/α_o subunit cDNA are highly homologous in their coding sequences but vary greatly in their untranslated sequences (22), the antisense constructs in both α_o and α_i2 antisense constructs included the full-length of the coding sequences, including 300 to 800 base pairs of 5' (α_o antisense construct) or 3' (α_i2 antisense construct) untranslated region to increase the specificity of ablation of the gene expression of

each α subunit. The resulting α_o antisense construct blocks the gene expression of both α_{oA} and α_{oB} subunits because the two forms of α_o subunits mainly vary at 3'untranslated region which was not included in the α_o antisense construct (26,27). In order to achieve the highest expression of the antisense RNA's to complete ablation of α_o or α_i2 subunits, a large amount of plasmid DNA (200-300 μg/10 cm^2 dish) was used in transfection. More than 5% of cells were stably transfected after three weeks of selection in 150 μg/ml of hygromycin B. Since hygromycin B is cytotoxic, it kills the cells with the lower level of expression of PY-3 plasmid at higher concentration. Thus, the concentration of hygromycin B was raised to 250-400 μg/ml which allowed us to select the clones with the highest resistance to hygromycin B and, consequently, expressing the highest levels of the antisense RNA.

Using this two-step selection procedure, twelve clones from each transfection were selected and characterized. Analysis of RNA from α_o antisense transfectant clones using reverse transcription-polymerase chain reaction (RT-PCR) with a pair of oligonucleotides designed from 5' untranslated region and specific for α_{oA} and α_{oB} cDNA's indicated complete ablation of mRNA's of both forms of α_o subunits (data not shown). Analysis of RNA from α_i2 antisense clones by RT-PCR using a pair of primers specific for α_i2 cDNA indicated partial ablation of α_i2 gene expression (data not shown). Membranes prepared from the transfectants were analyzed by Western blot using antibodies specific for α_o or α_i2. The blots were enhanced by Masterscan images by which the weaker bands were visualized. Similar to the data obtained from RT-PCR analysis, the α_o subunits were undetectable in GoZR7-1,-2, and -3 (Fig 3A, lanes 2-4), and in GoD2L-8, -9, -11 clones (Fig.3B, lanes 2-4). The α_i2 subunits were essentially depleted in Gi2ZR7-5 (Fig 3B,C, lane 2) and Gi2D2L-7 (Fig. 3B,C, lane 3) clones. In the cells

transfected with pcDNA vector and PY-3 plasmid, the amount of α_o and α_i2 was not different from that in the non-transfected cells (data not shown).

To examine the extent of cross-hybridization of α_o and α_i2 antisense RNA's with sense RNA of other α subunits, membranes from several α_o antisense clones were probed with an antibody common to α_i subunits. No change in the level of α_i subunits in these clones was observed (Fig. 3B, lane 2-4). Furthermore, membranes from the α_i2-antisense clones were probed with antibody to α_i3 and showed no change in α_i3 subunits (Fig.3D, lanes 2-3). Moreover, the levels of ß subunits in both α_o and α_i2 depleted clones were not different from control cells (data not shown).

Ablation of receptor-mediated inhibition of calcium entry in α_o-depleted clones. The inhibition of the Bay K 8644-induced increase $[Ca^{2+}]i$ induced by D_{2S} receptors (in GoZR7 cells) was completely blocked (Fig. 4A). Notably, the dopamine D_{2L} receptor (in GoD2L cells) retained 30% of its activity in inhibiting the Bay K 8644-induced increase in $[Ca^{2+}]_i$ (Fig. 4B). Inhibition induced by somatostatin and carbachol was also completely abolished in both GoD2L (Fig. 4C and 4D) and GoZR7 (Table 2). While incomplete inhibition of the dopamine D_{2L} response was observed in several α_o antisense clones with undetectable α_o, whereas the ablation of the D_{2S} response was complete even in a clone with only about 70% reduction of α_o proteins. By contrast, in the α_i2-antisense clones, the inhibitory actions of each of the above receptors on Bay K 8644-induced calcium influx remained as effective as in control cells (Table 2). These results show that α_o is crucial for the PTX-sensitive inhibition of calcium influx, while α_i2 plays apparently no essential role in this response.

Alteration in receptor-mediated inhibition of cAMP accumulation in α_i2-depleted

clones. The effects of α_o or α_i2 knockout on receptor-mediated cAMP accumulation were examined in both unstimulated and VIP-stimulated GH4C1 cells. In the α_o-antisense clones, activation of somatostatin, muscarinic M4, dopamine D_{2S} or D_{2L} receptors fully retained their ability to inhibit cAMP accumulation (Fig. 5A and 5B), indicating that G_o is not involved in PTX-sensitive inhibition of adenylyl cyclase by any of these receptors. On the contrary, in the α_i2-antisense clones, receptor-induced inhibition of basal and VIP-stimulated cAMP accumulation were clearly impaired (Fig. 5C and 5D). All four receptors failed to inhibit basal cAMP accumulation, indicating a primary role of α_i2 in the regulation of basal adenylyl cyclase activity in GH4C1 cells. There were, however, discrepancies in the ability of each receptor to inhibit VIP-stimulated cAMP accumulation in the α_i2 antisense clones: inhibition via dopamine D_{2L} or muscarinic M4 receptor was completely abolished, while inhibition via dopamine D_{2S} receptors was retained by about 70%. Paradoxically, somatostatin potentiated VIP-stimulated cAMP accumulation by 30% in the α_i2 depleted clones. This potentiation was prevented by PTX pretreatment of the cells (data not shown).

Discussion

PTX-sensitive G_i/G_o proteins mediate the coupling of a large number of receptors for hormones and neurotransmitter to various second messenger-generating effectors, such as adenylyl cyclase (inhibition), calcium and potassium channels (inhibition and/or stimulation) and phospholipase C (stimulation). In order to analyze the specificity of G proteins in receptor-effector coupling in intact cells, the antisense technique leading to depletion/elimination of specific G protein subunits is the most powerful tool. For example, with this technique, the role of specific G protein subunit combinations in coupling of muscarinic M4 and somatostatin

receptors to inhibition of voltage-sensitive calcium channels in GH3 cells has been elucidated (17,28,29). In GH4C1 cells, PTX-sensitive G proteins couple to endogenously expressed somatostatin and muscarinic M4 receptors, as well as transfected dopamine D_2 and 5-HT1A receptors to inhibition of adenylyl cyclase and voltage-sensitive calcium channels activities (8,30). The aim of the present study to analyze the specificity of the α subunits of G_o and G_i2 in the coupling of various receptors to inhibition of cAMP synthesis and calcium entry. Therefore, α subunits of G_o and G_i2 were essentially or completely eliminated in GH4ZR7 and GH4D2L cells, expressing dopamine D_{2S} and D_{2L} receptors, respectively, by stable transfection of the full-length antisense constructs. The results demonstrate that α_o and α_i2 subunits were stably and specifically depleted using this transfection strategy without obvious changes in other G protein subunits. The specificity of this approach suggests they may be applied to knockout any target RNA product of interest.

Several conclusions emerge from the functional analysis of the antisense transfected cells. First, each of the four receptors studied couples to α_o but not to α_i2 to inhibit calcium entry via dihydropyridine-sensitive calcium channels. The α_o subtype (α_{oA} and α_{oB}) specificity was not addressed in the present study, because the α_o antisense construct used ablates the expression of both isoforms of α_o. In contrast to the dopamine D_{2S}, somatostatin and muscarinic M4 receptors, dopamine D_{2L} receptor-mediated inhibition of calcium entry was only partially abrogated in α_o-depleted cells. Since the action of the dopamine D_{2L} receptor is completely blocked with PTX pretreatment, it is feasible to assume that in the α_o-depleted clones dopamine D_{2L} receptors may recruit remaining α_i subunits to partially retain inhibition of calcium entry in the αo-depleted clones. However, mediation by an undetectable amount of α_o proteins present

in the α_o antisense clones cannot be excluded. Nevertheless, the data indicate that dopamine D_{2S} and D_{2L} receptors differ in G protein-mediated coupling to calcium channels.

Second, whereas α_o apparently couples receptors to inhibition of calcium channel activity, this α subunit appears to be dispensable for receptor-induced inhibition of adenylyl cyclase. By contrast, this receptor response was clearly altered in α_i2-depleted cells. Inhibition of VIP-stimulated cAMP synthesis was abrogated for dopamine D_{2L}, muscarinic M4 and somatostatin receptors. Remarkably, in the α_i2-depleted cells, activation of somatostatin receptors potentiated the cAMP elevating action of VIP receptors. In contrast, the inhibitory action of dopamine D_{2S} receptor was largely retained in the α_i2-depleted clones. Since both the stimulatory action of somatostatin and the inhibitory action of dopamine D_{2S} receptors was PTX-sensitive, it can be concluded that in the α_i2-depleted clones, somatostatin and dopamine D_{2S} receptors may couple to remaining G_i and/or G_o proteins. Most likely, the dopamine D_{2S} receptor couples to G_i1 and/or G_i3 to inhibit VIP-stimulated cAMP formation in α_i2-depleted cells. On the other hand, conditional stimulation of adenylyl cyclase by somatostatin in α_i2-depleted clones may be mediated by released $\beta\gamma$ dimers. To date, six different subtypes of adenylyl cyclase have been cloned and at least two additional isoforms of the enzyme have been shown to be present (31,32). Whereas all six cloned adenylyl cyclases can be stimulated by α_s, only types II and IV were stimulated by free $\beta\gamma$ dimers upon activation of G_s either *in vitro* or *in vivo* (33,34). Indeed, preliminary results indicate that type II adenylyl cyclase is expressed in GH4C1 cells (Y.F. Liu unpublished observation). The reason that potentiation of cAMP synthesis was only seen upon activation of the somatostatin receptor, but not of the muscarinic M4 or the two D_2 receptors, is not addressed here. One possibility is that these receptors are associated with G

proteins containing different combinations of α, ß and γ subunits, as reported for somatostatin and muscarinic M4 receptor coupling to calcium currents in GH3 cells (28,29), and that only certain pairs of ßγ dimers can stimulate adenylyl cyclase.

Third, a difference in G protein specificity for inhibitory receptor action on basal and stimulated cAMP accumulation was apparent. While inhibition of basal cAMP accumulation by any of the four receptors studied was abrogated in α_i2-depleted cells, the inhibitory action of activated dopamine D_{2S} receptors on VIP-stimulated cAMP formation was largely retained. Thus, different receptors may regulate different levels (basal vs. G_s-stimulated) of cellular adenylyl cyclase activity by coupling to distinct α_i-adenylyl cyclase pairs.

Fourth, the data presented provide compelling evidence that the two alternatively spliced forms of dopamine D_2 receptors differ in their association to G_o and G_i proteins. Both with regard to inhibition of calcium entry via dihydropyridine-sensitive calcium channels and VIP-stimulated cAMP accumulation, the two receptors exhibited different actions in α_o and α_i2-depleted cells, while no clear difference was detected in the non-depleted parental cells. The differences between D_{2S} and D_{2L} receptors did not appear to be due to the extent of α subunit depletion in different antisense clones because the D_{2L} receptor-mediated calcium response was observed in complete α_o-depleted clones whereas the D_{2S} response was not detected even in incomplete α_o-depleted clones. A differential sensitivity of the two forms of dopamine D_2 receptors to inhibitory modulation by protein kinase C has been reported (35). As the two forms of dopamine D_2 receptors differ within the third cytoplasmic loop, the data presented support the notion of the importance of this domain in receptor coupling to G proteins (36-39).

While injection of antibodies or oligonucleotides is largely limited to single cell

preparations for electrophysiological measurements. the stable antisense transfection approach used here allows construction of cell lines to verify the completion and specificity of the ablation of gene expression and is particularly useful when large amounts of cell preparations are required. In the present study, G protein α_o and α_i2 were specifically and essentially depleted from GH4C1 cells. This allowed simultaneous dissection of the role of these two PTX-sensitive G protein α subunits in linking of various receptors to two effectors mechanisms. Further studies on various G proteins subunits are in progress to analyze the roles of distinct G protein subunits in receptor transmembrane signalling in intact cells.

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Table 1. Relative efficacies of inhibitory responses induced by various receptors in GH4C1 clones. Values represent percent inhibition of Bay K 8644-induced peak in $[Ca^{2+}]_i$ or VIP-increased cAMP level for each compound, normalized to the percent inhibition obtained with carbachol (defined as 1), and represent mean ± SD of at least four independent determinations.

	GH4ZR7		GH4D2L	
	$[Ca^{2+}]_i$	cAMP	$[Ca^{2+}]_i$	cAMP
Carbachol	1	1	1	1
Somatostatin	1.05 ± 0.01	1.14 ± 0.02	1.01 ± 0.032	1.22 ± 0.03
Dopamine	1.41 ± 0.05	1.44 ± 0.07	1.46 ± 0.06	1.48 ± 0.04

Table 2. Effects of elimination of α_o or α_i2 on receptor-mediated inhibition of calcium entry. Data are presented as mean ± SD of three independent experiments in which the actions of dopamine (DA, 1 μM), carbachol (CAR, 10 μM), and somatostatin (SS, 0.25 μM) on the Bay K 8644 (BAY, 1 μM)-induced increase in $[Ca^{2+}]_i$ were measured. Values are expressed as -fold basal level of $[Ca^{2+}]_i$, and a value of unity (1) indicates no change in the basal level of $[Ca^{2+}]_i$.

	GH4ZR7	GoZR7	GoD2L-8	Gi2ZR7	Gi2D2L
BAY K8644	2.40 ± 0.22	2.51 ± 0.13	2.60 ± 0.17	2.55 ± 0.21	2.44 ± 0.16
CAR + BAY	1.33 ± 0.09	2.52 ± 0.22	2.56 ± 0.25	1.36 ± 0.07	1.38 ± 0.05
SS + BAY	1.30 ± 0.06	2.65 ± 0.24	2.71 ± 0.28	1.34 ± 0.14	1.31 ± 0.07
DA + BAY	1	2.49 ± 0.14	1.76 ± 0.15	1	1
DA + BAY*	2.43 ± 0.11	2.48 ± 0.14	2.56 ± 0.26	2.57 ± 0.19	2.48 ± 0.17

* The cells were pretreated with PTX (50 ng/ml) overnight.

Figure legends

Fig. 1. Inhibition of cAMP accumulation by various receptor agonists in GH4ZR7 and GH4D2L cells. The influence of dopamine (DA, 1 μM), somatostatin (SST, 0.25 μM) and carbachol (CAR, 10 μM) on basal and VIP (0.2 μM)-stimulated cAMP levels was studied as described in "Experimental Procedures" in GH4ZR7 (**A**) and GH4D2L (**B**) cells. Data averaged from three independent experiments done in triplicate are presented as mean $\pm$ SD.

Fig. 2. Inhibition of calcium entry by various receptors in GH4ZR7 and GH4D2L cells. The influence of dopamine (DA, 1 μM) in GH4ZR7 cells (**A**), and of dopamine (DA, 1 μM) (**B**), carbachol (CAR, 10 μM) (**C**) and somatostatin (SS, 0.25 μM) (**D**) in GH4D2L cells on Bay K 8644 (BAY, 1 μM)-induced increase in $[Ca^{2+}]_i$ was determined as described in "Experimental Procedures". The curves were done in duplicate and were repeated at least three times with similar results. The receptor agonists were added to cuvette 1-2 min prior to recording, and Bay K 8644 was added as indicated.

Fig. 3. Alterations in membrane level of α_o and α_i subunits in the α_o and α_i2 antisense transfectants. **A.** Western blot analysis of control (GH4ZR7, lane 1) and α_o antisense transfectant membranes (GoZR7-1, lane 2; GoZR7-2 lane 3; GoZR7-3, lane 4). The blots were probed with antibody specific to α_o. **B.** Western blot analysis of control (GH4D2L, lane 1), and α_o antisense transfectant membranes (GoD2L-8, lane 2; GoD2L-9, lane 3; GoD2L-11, lane 4). The blot was probed with an antibody specific for α_o. **C.** Western blot analysis of control (GH4D2L, lane 1), and α_i2 antisense transfectant membranes (Gi2ZR7-5, lane -5; Gi2D2L-7, lane 3). The blots were probed with antibody specific to α_i2. **D.** Western blot analysis of control (GH4D2L, lane 1) and α_o transfectant membranes (GoD2L-8, lane 2; GoD2L-9, lane 3;

GoD2L-11, lane 4). The blot was probed with an anti-α_i common antibody. D. Western blot analysis of control (GH4ZR7, lane 1) and α_i2 antisense transfectant membranes (Gi2ZR7-5, lane 2; Gi2D2L-7, lane 3). The blot was probed with an antibody specific for α_i3. The blot shown in parts A to C were enhanced by Masterscan images. Preparation of the various clones and Western blot analysis were performed as described in "Experimental Procedures".

Fig. 4. Blockade of receptor-induced inhibition of calcium entry in α_o antisense clones. The influence of dopamine (DA, 1 μM) in GoZR7 cells (**A**), and of dopamine (DA, 1 μM) (**B**), carbachol (CAR, 10 μM) (**C**), and somatostatin (SS, 0.25 μM) (**D**) in GoD2L cells on Bay K8644 (BAY, 1 μM)-induced increase in $[Ca^{2+}]_i$ was determined as described in the legend to Fig. 2. **Fig. 5. Alteration in receptor-induced inhibition of cAMP accumulation in α_o and α_i2 antisense clones.** The influence of dopamine (DA, 1 μM), somatostatin (SS, 0.25 μM), and carbachol (CAR, 10 μM) on basal cAMP levels and cAMP levels stimulated by VIP (0.2 μM) was determined in α_o (GoZR7, **A**; GoD2L, **B**) and α_i2 (Gi2ZR7, **C**; Gi2D2L, **D**) antisense clones as described in the legend to Fig. 1.

FIG. 1A

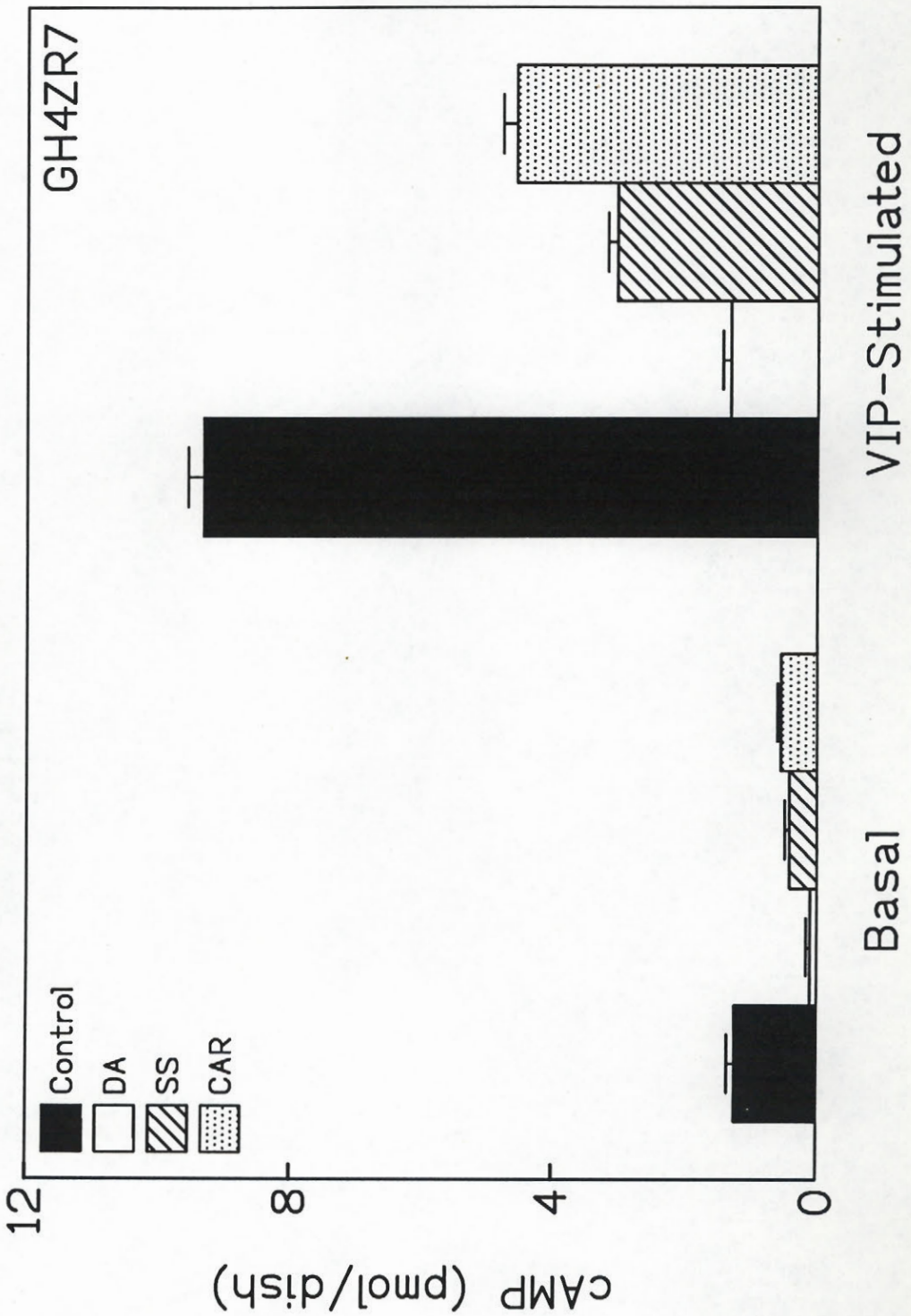
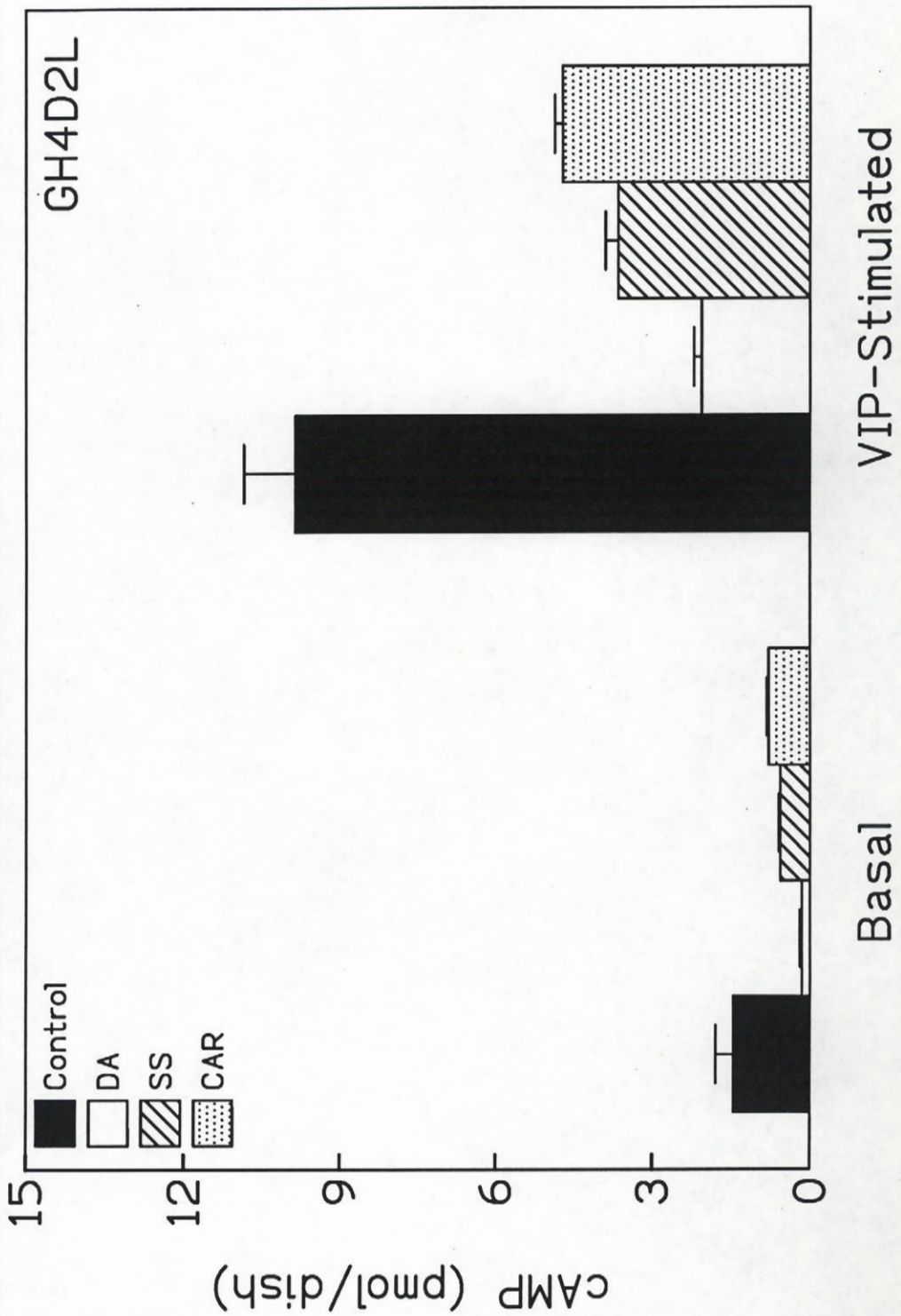

GH4ZR7
Control
DA
SS
CAR
12
8
4
0
cAMP (pmol/dish)
Basal
VIP-Stimulated

FIG. 1B


GH4D2L
Control
DA
SS
CAR
15
12
9
6
3
0
cAMP (pmol/dish)
Basal
VIP-Stimulated

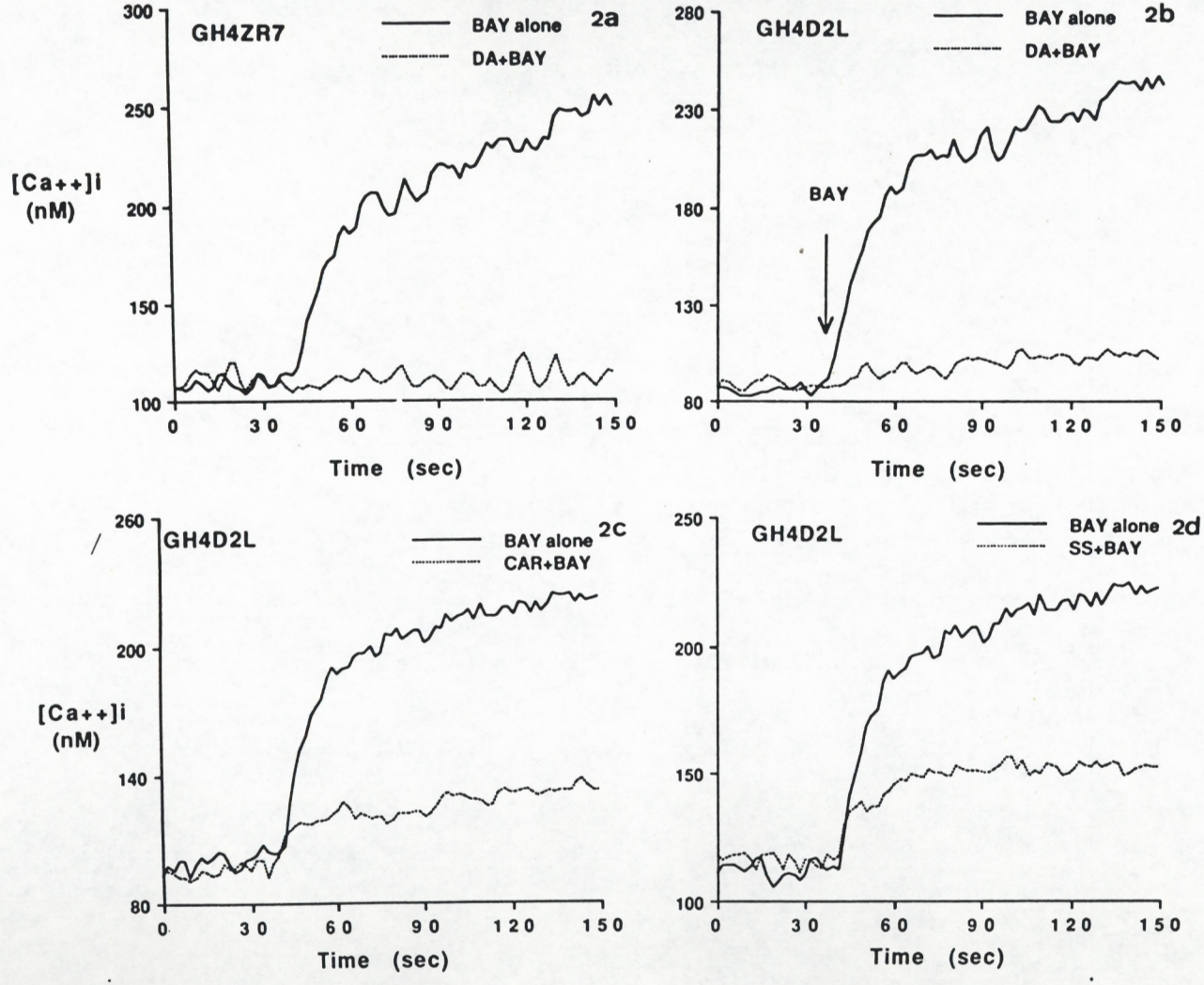

GH4ZR7
BAY alone
DA+BAY
2a
[Ca++]i
(nM)
300
250
200
150
100
0
30
60
90
120
150
Time (sec)
GH4D2L
BAY alone
DA+BAY
2b
BAY
280
230
180
130
80
0
30
60
90
120
150
Time (sec)
GH4D2L
BAY alone
CAR+BAY
2c
[Ca++]i
(nM)
260
200
140
80
0
30
60
90
120
150
Time (sec)
GH4D2L
BAY alone
SS+BAY
2d
250
200
150
100
0
30
60
90
120
150
Time (sec)

FIG. 2A-2D

FIG. 3A

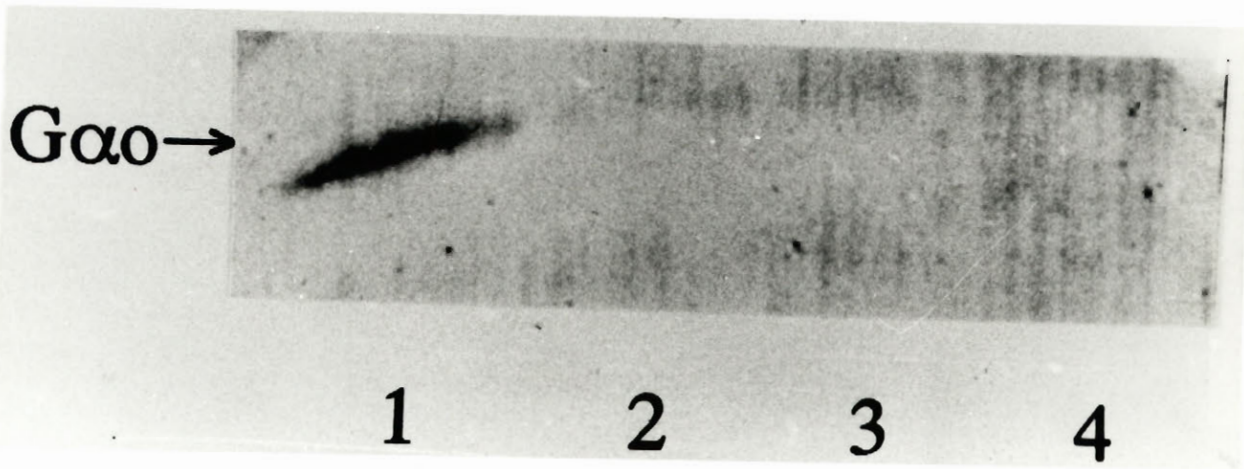


FIG. 3B

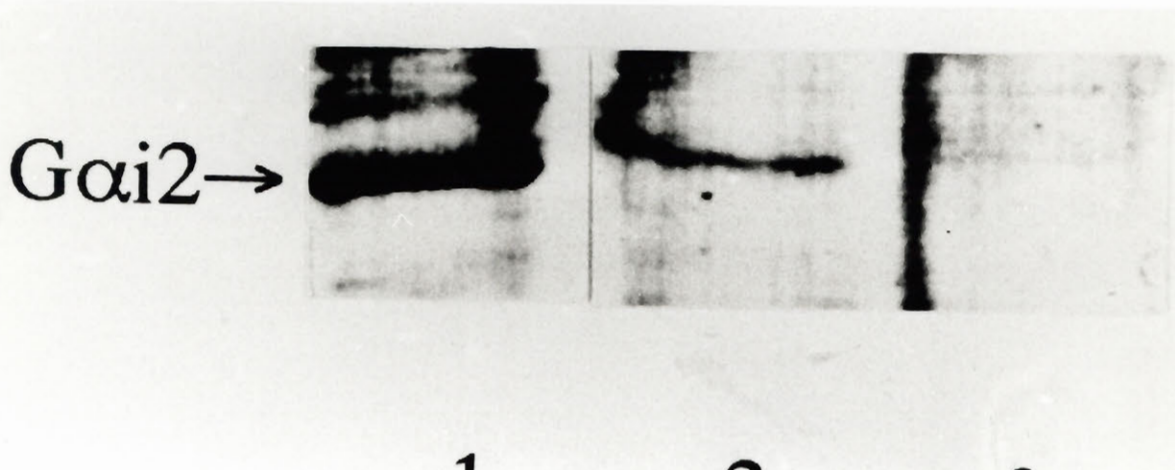


FIG. 3C

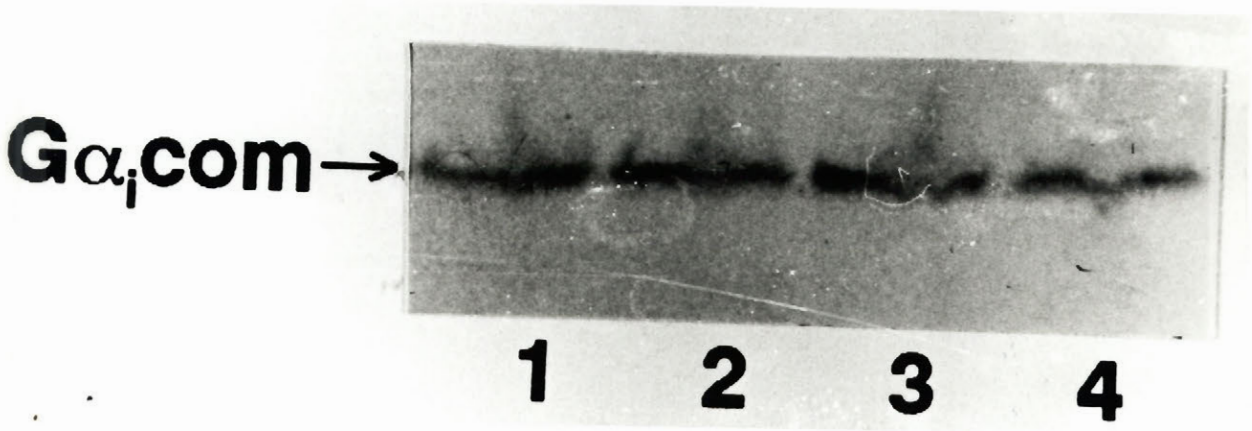
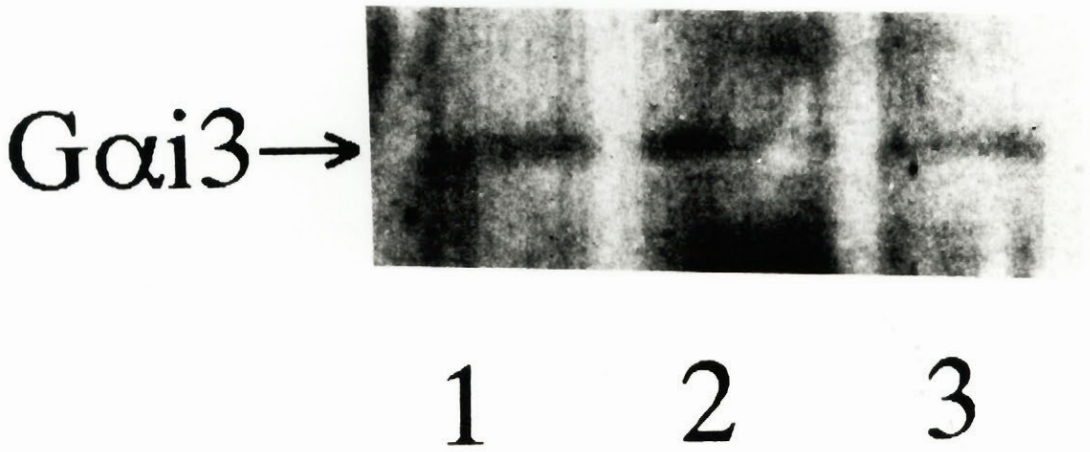


FIG. 3D



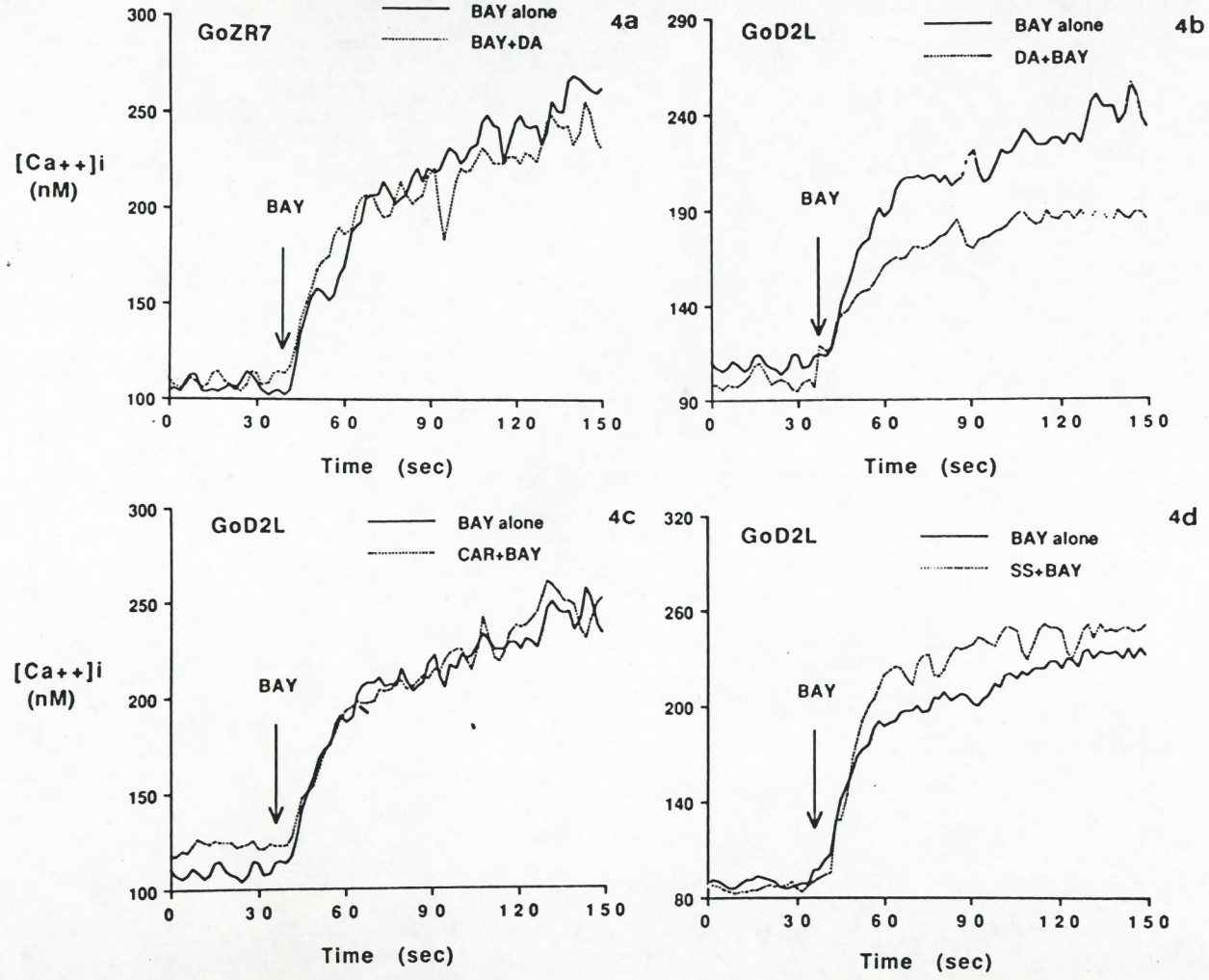

GoZR7
BAY alone
BAY+DA
4a
[Ca++]i
(nM)
BAY
Time (sec)
GoD2L
BAY alone
DA+BAY
4b
BAY
Time (sec)
GoD2L
BAY alone
CAR+BAY
4C
[Ca++]i
(nM)
BAY
Time (sec)
GoD2L
BAY alone
SS+BAY
4d
BAY
Time (sec)

FIG. 4A-4D

FIG. 5A

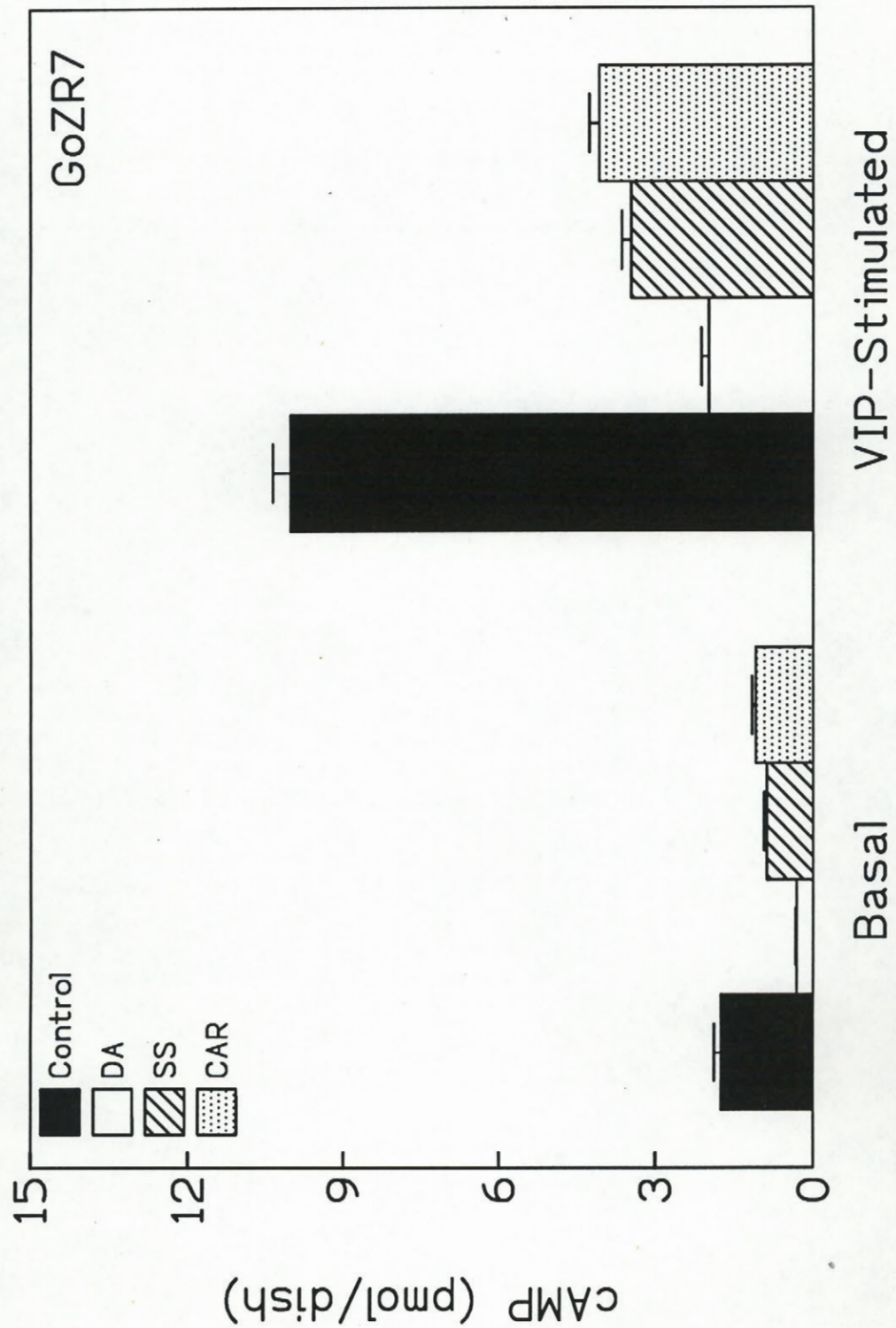

GoZR7
Control
DA
SS
CAR
15
12
9
6
3
0
cAMP (pmol/dish)
Basal
VIP-Stimulated

FIG. 5B

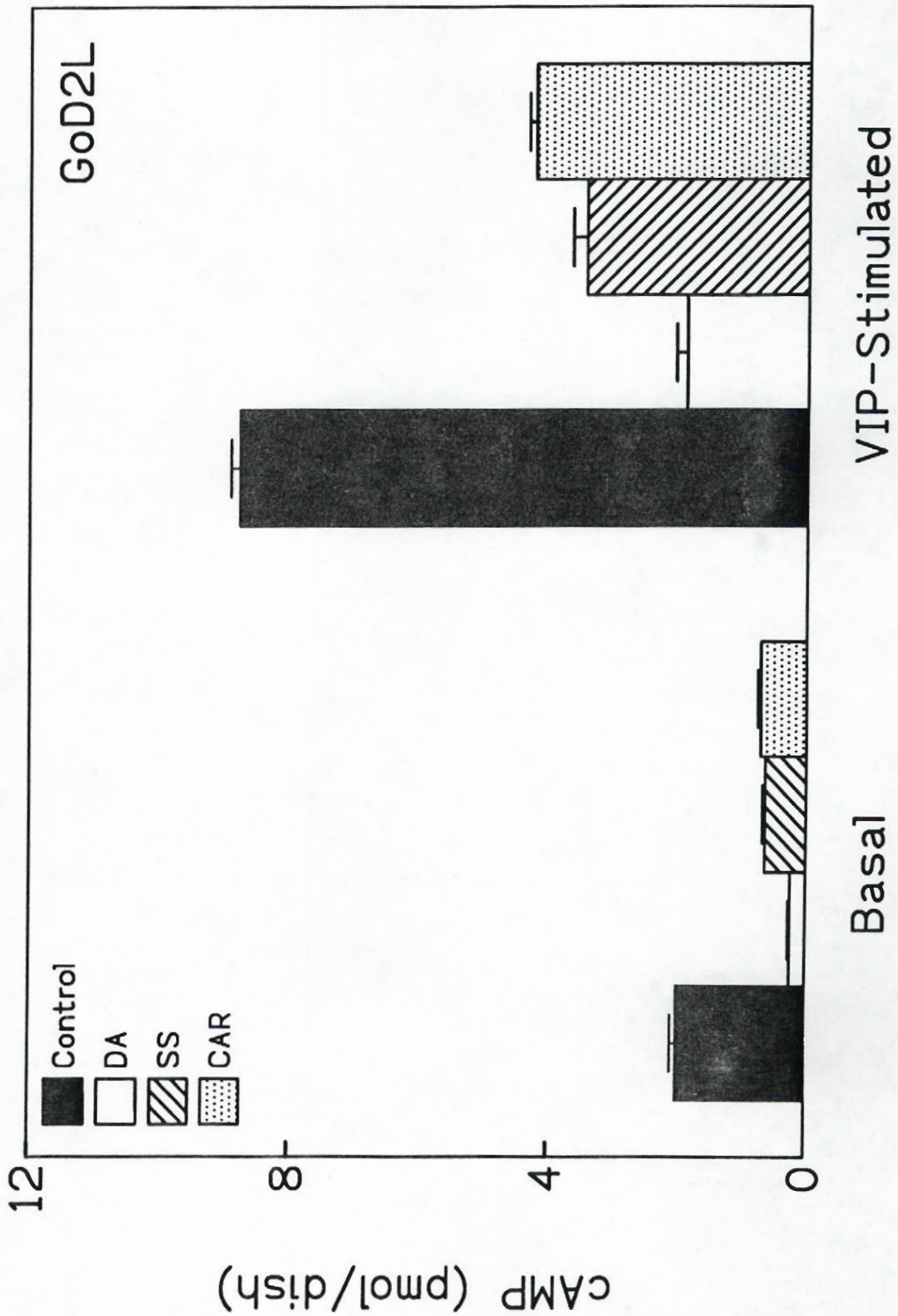

GoD2L
Control
DA
SS
CAR
12
8
4
0
cAMP (pmol/dish)
Basal
VIP-Stimulated

FIG. 5C

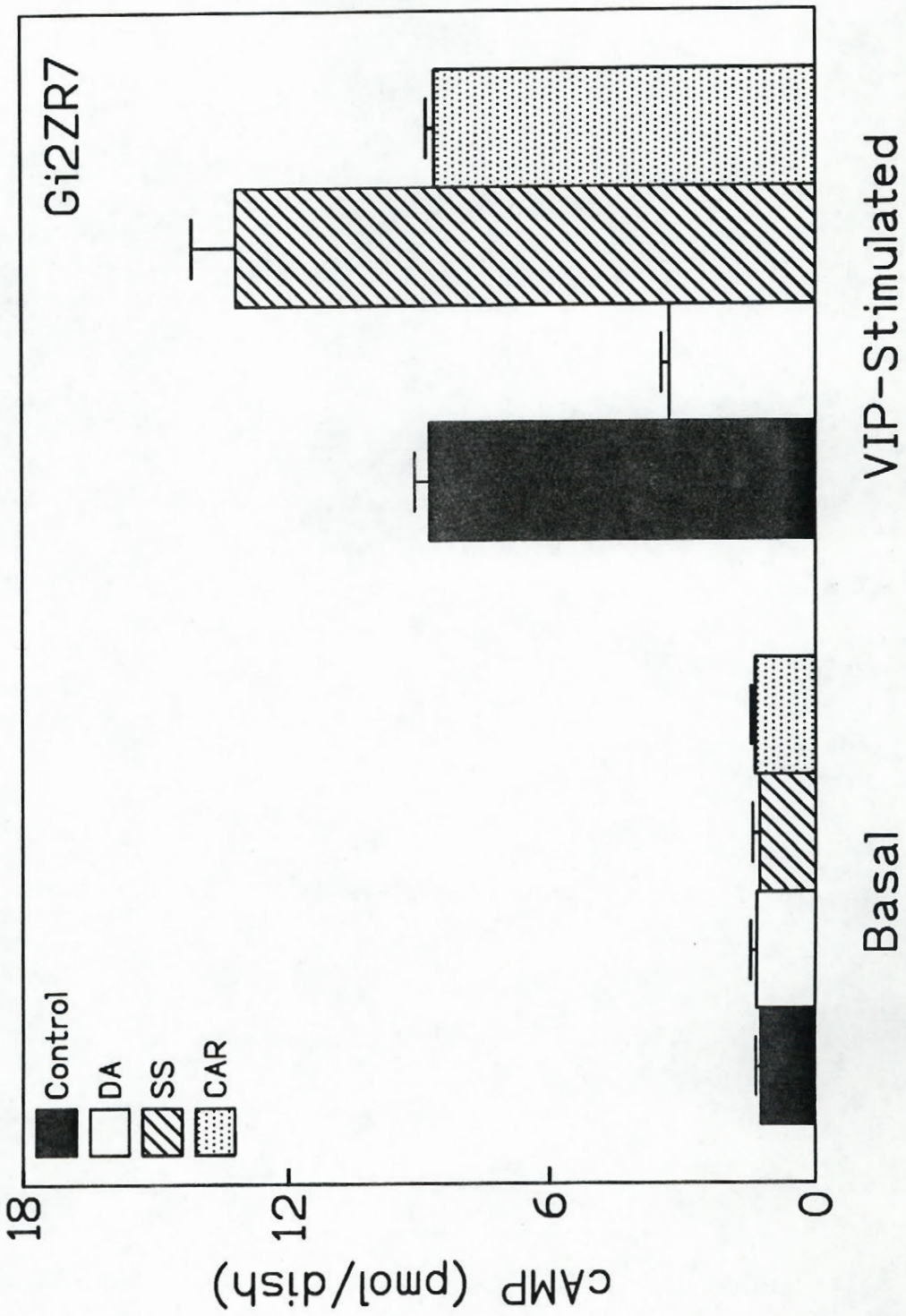
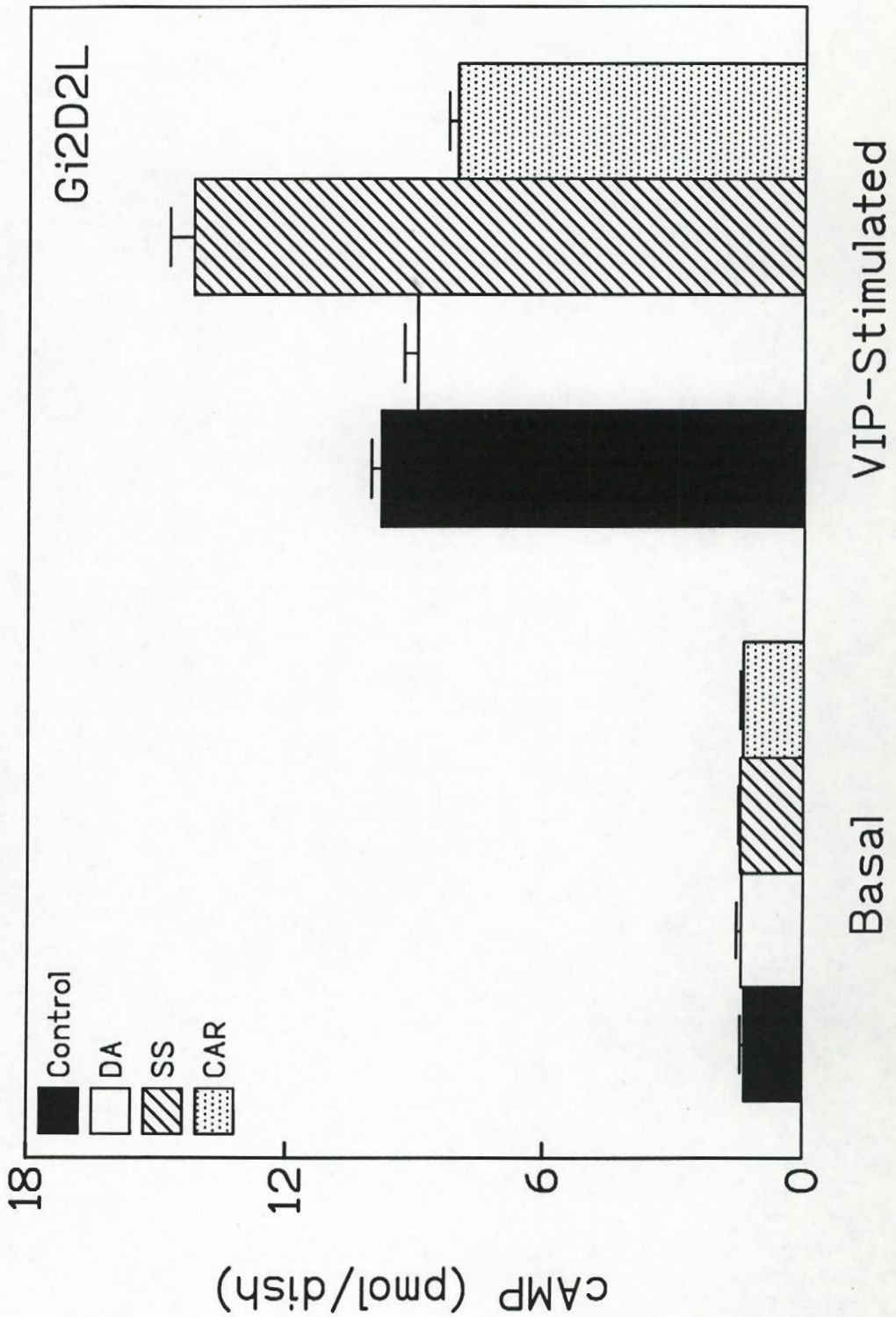

Gi2ZR7
Control
DA
SS
CAR
18
12
6
0
cAMP (pmol/dish)
Basal
VIP-Stimulated

FIG. 5D


Gi2D2L
Control
DA
SS
CAR
18
12
6
0
cAMP (pmol/dish)
Basal
VIP-Stimulated

CHAPTER VII GENERAL DISCUSSION

CHAPTER VII GENERAL DISCUSSION

The present studies were undertaken to further investigate the biochemical mechanisms of transmembrane signalling of several G protein coupled receptors, including 5-HT1A, dopamine-D1, -D2S and D2L receptors. Essentially, we are interested in the cell-specificity of receptor-induced signals and the modulatory roles of protein kinase A and C on each of different signal transduction pathways elicited by these receptors. The specificity of distinct G proteins in the coupling of these receptors to multiple effector systems was also investigated.

Summary of the Results:

1. Cell-specific signalling of the 5-HT1A, dopamine-D2S, -D2L and -D1 receptors

When expressed in rat GH4C1 pituitary cells, the 5-HT1A, D2S and -D2L receptors, via PTX-sensitive G proteins, Gi/Go, induced inhibition of both basal and VIP-stimulated adenylyl cyclase activity and a decrease in the conductance of voltage dependent calcium channels. The inhibitory actions induced by D2S or D2L receptors were always greater than those induced by 5-HT1A receptors, suggesting that there are differences in molecular interactions of these receptors with Gi/Go. By contrast, activation of the dopamine-D1 receptors stimulated adenylyl cyclase and increased the conductance of voltage-dependent calcium channels by coupling to the CTX-sensitive G protein, Gs. The increase of calcium entry induced by the D1 receptor was mediated by the increase in intracellular cAMP levels to activate PKA (Table 5).

Unexpectedly, when expressed in Ltk- fibroblast cells, the rat 5-HT1A, and human D2S and D2L receptors, all induced an increase in [Ca++]i and stimulation of PLC. Both increase in [Ca++]i and stimulation of PLC by the 5-HT1A, D2S and D2L receptors were agonist

concentration-dependent; EC50 for stimulation of PLC for each receptor was correlated with IC50 of the receptor-mediated inhibition of adenylyl cyclase; and was blocked by selective antagonists of each receptor. Furthermore, activation of PLC was not due to over-expression of these receptors because similar amounts of receptors (about 1 pmol/m protein) were also expressed in GH4C1 cells (see Chapter III & IV) which lack this PLC activation pathway. Since basal activity of adenylyl cyclase was not altered by the 5-HT1A, D2S and D2L receptors, the PI signal was the major signal elicited by these receptors in Ltk- cells (Table 5).

2. Selective modulation of receptor signalling by PKA and PKC

In both GH4C1 pituitary and Ltk- fibroblast cells, activation of PKA or PKC differentially modulated signal transduction pathways elicited by each receptor examined (Table 6). Activation of PKC inhibited the novel stimulation of PLC and increase in [Ca++]i induced by the 5-HT1A, D2S, and D2L receptors. While activation of PKA alone had no effect on the receptor-induced PI signal but potentiated the inhibitory action of PKC. PKC had no effect on receptor-mediated inhibition of adenylyl cyclase in both GH4C1 and Ltk- fibroblast cells (Table 6). Interestingly, the D2L receptor-mediated PI signal was highly resistant to desensitization by PKC, suggesting the presence of differential modulation of transmembrane signalling of the dopamine-D2S and -D2L receptors by PKC.

The actions of PKA and PKC on the D1 receptor-mediated responses were different from their actions on the responses induced by Gi/Go coupled receptors, eg. 5-HT1A, D2S and D2L receptors. In GH4C1 cells, the D1 receptor-induced increase of the conductance of voltage-dependent calcium channels was mediated by PKA; while in Ltk- cells, prior activation of PKA remarkably enhanced the D1 receptor-induced stimulation of PLC and increase in [Ca++]i,

which were inhibited by PKC pre-activation (Table 6).

3. The specificity of Go and Gi2 in coupling of hormone receptor to multiple signalling systems

By modifying stable transfection method, we were able to specifically and completely deplete distinct G protein α subunits from rat pituitary GH4C1 cells by over-expressing their cognate antisense RNA's. The different antisense transfectants (Table 7) were utilized to study the roles of αi/αo subunits in coupling of dopamine-D2S, -D2L, somatostatin, muscarinic-M4 receptors to inhibition of adenylyl cyclase and closure of calcium channels.

Elimination of αo did not alter receptor-mediated inhibition of cAMP accumulation, but abolished receptor-mediated closure of calcium channels; only the action of D2L receptor-mediated inhibition of calcium influx was partially retained when αo subunits were eliminated, suggesting that D2L receptor may be able to recruit Gi proteins to inhibit calcium entry. By contrast, depletion of αi2 did not alter receptor-mediated closure of calcium channels but impaired receptor-mediated inhibition of cAMP accumulation: all four receptor-mediated inhibition of basal cAMP synthesis and D2L- or M4-receptor-mediated inhibition of VIP-stimulated cAMP synthesis were completely abolished; however, the action of D2S receptors on VIP-stimulated cAMP synthesis remained unaltered; while somatostatin receptors potentiated the action of VIP, suggesting that different Gi/Go coupled receptors might use different α subunit to inhibit basal and Gs-stimulated adenylyl cyclase activity. This hypothesis is further supported in αi- or αi3 eliminated clones. In αi3-eliminated clones, activation of the D2S receptors increase basal level of cAMP accumulation while inhibiting VIP-stimulated cAMP accumulation; whereas in αi1-eliminated clones, activation of the 5-HT1A receptor increase basal cAMP

accumulation but inhibited VIP-stimulated adenylyl cyclase activity (Table 8).

Taken together, the above results indicate that αo specifically triggers closure of calcium channels, whereas αi2 specifically initiates inhibition of adenylyl cyclase. From the present experiments, each receptor tested can interact with more than one α subunits, but different receptors used distinct α subunits to regulate different effector activity. The interaction of receptor to αi is subject to be altered when a Gs coupled receptor is activated, suggesting that the coupling of hormone receptor and Gi proteins is a dynamic process. Moreover, although the signals induced by dopamine D2S is similar to these induced by the D2L receptors in both GH4C1 and Ltk- cells, there are clear differences in interaction with Go and Gi2 between these two form of the dopamine D2 receptors.

Discussion

I. Cell-Specific Signalling of Hormone Receptors

In neurons, the 5-HT1A, dopamine D2S and D2L receptors act as inhibitory receptors, and they induce inhibition of adenylyl cyclase, closure of calcium channels and opening of potassium channels (Frazer et al, 1990; Civelli et al 1993; Sibley et al 1992). As illustrated in Fig. 1, the 5-HT1A, D2S and D2L receptors induce inhibition of cAMP accumulation as well as calcium entry when expressed in GH4C1 pituitary cells. Apparently, these receptors exhibit similar signalling phenotype as was found in the CNS where these receptors normally exist (Civelli et al, 1993). The dopamine D1 receptors are also prominently expressed in neuronal cells (Civelli et al, 1993). When expressed in GH4C1 cells, the D1 receptor also exhibits similar signalling phenotypes as observed in neurons: stimulation of adenylyl cyclase and increase of calcium influx.

In Ltk- fibroblast cells, all receptors examined exhibited different signalling phenotypes from that observed in neurons: the signalling phenotype of "inhibitory receptors" (5-HT1A, D2S and D2L receptors) was switched to that of a "stimulatory receptor" and induced stimulation of PLC to release ionomycin-sensitive calcium pools (Berridge, 1993). The D1 receptors also induced a small but significant increase in [Ca++]i by activation of PLC when expressed in Ltk- cells. More interestingly, the weak PI signal induced by the D1 receptor was markedly amplified when PKA was preactivated. Since PKA activation actually inhibits the D1-receptor-mediated stimulation of adenylyl cyclase (Sidhu et al, 1991), the stimulation of PLC pathway may become the predominant signal transduction pathway of the D1 receptor after the cAMP signal is turned off.

It was not surprising that all receptors examined exhibited a similar signalling phenotype in GH4C1 cells to that found in neurons. Although GH4C1 cells are endocrine cells, they share many characteristic features for G protein signalling with neuronal cells as discussed in Chapter I. First, they are both electrically excitable cell types which express voltage-dependent calcium channels and potassium channels (Lldeo et al, 1990; Sibley et al, 1992). Second, as in neurons, where many different G protein coupled receptors are co-expressed, activation of these receptors regulate neurotransmitter synthesis, storage, release. GH4C1 cells express prolactin and growth hormone whose synthesis, storage and secretion are also regulated by G protein coupled receptors, eg., somatostatin, muscarinic-M4, VIP and TRH receptors (Dorflinger & Schonbrunn 1983; Yatani et al, 1987; Enyeart et al, 1990). Furthermore, $G\alpha o$ and $G\alpha i$ are mainly expressed in neuronal cells, they are also strongly-expressed in GH4C1 cells; $G\gamma 3$ and $G\gamma 5$ subunits are dominantly expressed in neurons, they are also found in GH4C1 cells (Gautum et al, 1990;

Kleuss et al, 1993). The similarity in expression of G proteins and effectors as well as the analogous function of G protein coupled receptors in the regulation of neurotransmitter/hormone release in neurons and GH4C1 cells give the foundation of the similarity of G protein signalling.

On the other hand, the cellular milieu for G protein signalling in Ltk- fibroblast cells is very different from that in GH4C1 cells as well as in neurons. Ltk- cells are not electrically excitable cells. They do not express any of known voltage-dependent calcium channels and potassium channels (Liao et al 1990; Birnbaumer, L. personal communication), and application of 40 mM KCl does not alter intracellular calcium concentration (Albert, unpublished observation). Although a few stimulatory G protein coupled receptors are expressed in Ltk- cells, they do not have analogous function in the regulation of neurotransmitter/hormone release because Ltk- cells do not express hormones and lack a regulated secretory pathway. Furthermore, both Gαo and Gαi1 are absent in Ltk- cells (Y.F.Liu unpublished observations). Provided that proper G proteins and necessary effectors, eg. voltage-dependent calcium channels, potassium channels, and perhaps certain type of adenylyl cyclase (eg. Type II) are naturally absent in Ltk cells and are present in GH4C1 cells (See chapter I, Table 1), it was expected that the signal transduction pathways mediated by G protein coupled receptors should be different from those found in GH4C1 pituitary cells. It is plausible to expect that the receptors examined will induce cell-specific signals in GH4C1 pituitary and Ltk- fibroblast cells.

PTX-coupled receptor-mediated activation of PLC naturally occurs in neutrophils and HL-60 granulocytes (Kikuchi et al, 1986; Thomas et al 1991). It is also found in several other cell systems transfected with a variety of the cloned PTX-sensitive G protein coupled receptors. In Rat-1 fibroblast cells, transfection of α2-C10 receptors induced a PTX-sensitive stimulation

of PLC activity, and this signal appears to be mediated by both Gi2 and Gi3 (Milligan et al 1991). In CHO cells, transfection of M2- or M3-muscarinic receptors all induced activation of PLC; Gi2 and Gi3 are reported to be involved in M2- but not M3-muscarinic receptor-mediated stimulation of PLC (Dell'Acqua et al, 1993). In Hela cells, the transfected 5-HT1A receptor induced stimulation of PLC, a pathway which is involved in the receptor-mediated phosphate uptake (Raymond et al 1990). In COS-7 cells, the transfected 5-HT1A receptors also mediated stimulation of PLC activity (Fargin et al 1989). Moreover, in Ltk- fibroblast cells, stimulation of the transfected rat D2S receptors increased PI turnover to release intracellular calcium stores (Vallar et al, 1990). These results suggest that most Gi/Go coupled receptors can couple to stimulation of PLC in appropriate cellular milieu.

At present, G proteins and the subtypes of PLC involved in PTX-sensitive stimulation of PLC remain unclear. Go has been shown to be involved in muscarinic-M2 receptor-induced calcium mobilization in Xenopus oocytes (Moriarty et al, 1990; Padrell et al 1991); while both Gi2 and Gi3 are implicated in α2-adrenergic or muscarinic-M2 receptor-mediated stimulation of PLC in rat-1 and CHO cells respectively. However, αo1, αo2, αi1, αi2, and αi3 all failed to stimulate any subtypes of known PLC *in vitro*, whereas q and α11 can effectively stimulate PLC activity *in vitro* (Wu et al, 1992). Recent studies indicate that in certain cell systems, such as HL-60 granulocytes, stimulation of PLC by PTX-sensitive G protein coupled receptors (f-MLP receptor) is mediated by ßγ dimers (Camps et al 1992; Katz et al, 1992; Park et al, 1993). Several studies have suggested that hormone receptor can select different ßγ dimers (Kleuss et al 1992; 1993). It remains to be addressed whether every combination of ßγ dimers can stimulate PLC-ß, and whether stimulation of PLC by PTX-sensitive pathways is universally

mediated by ßγ dimers. It is possible that PTX-sensitive G proteins stimulate an unidentified PLC isoform.

Pretreatment with PTX overnight completely blocked the action of the 5-HT1A, D2S or D2L receptors in Ltk- cells, suggesting that Gi/Go are involved in these responses elicited by these receptors. Using transient expression of distinct αi-antisense constructs, it was found that αi2, but not αi3 antisense constructs blocked the 5-HT-induced increase in [Ca++]i in L cells, suggesting Gi2 mediated the 5-HT1A receptor-induced PI signal (Y.F.Liu unpublished observation). This result is different from the results obtained with α2-C10 in rat-1 cells, and with M2 receptors in CHO cells (Milligan et al, 1991; Dell'Acqual et al, 1993). In these two studies, the activated Gi proteins were traced by CTX-mediated ADP-ribosylation based on CTX induced ADP-ribosylation of activated PTX substrates and both Gi2 and Gi3 were shown to be involved in α2 or M2 receptor-mediated PI signal. Different approaches may account for the different results between our studies and the others. However, the above experiments can not rule out the involvement of G protein ßγ dimers in the receptor-mediated PLC activity in Ltk- cells.

Activation of several Gs-coupled receptors has been found to stimulate both adenylyl cyclase and PI turnover in several cell systems. In CHO cells, activation of the transfected TSH receptors induces two independent signals, increase of cellular cAMP and stimulation of PI turnover (Van Sande et al 1992). In Ltk- cells, the transfected LH receptors are dually-coupled to the stimulation of adenylyl cyclase and PI turnover (Gudermann et al 1992). In COS-7 cells, stimulation of the transfected ß2-adrenergic receptor increased cAMP synthesis but not PLC activity, while activation of the transfected PTH receptors in COS-7 cells increased both cAMP

synthesis and PI turnover (Abou-Samra et al, 1992). It was found that the native PGE1 receptor in Ltk- cells did not induce increase in [Ca++]i and PI turnover (see Chapter IV). Taken together, certain Gs-coupled receptors, but not all, can induce both stimulation of adenylyl cyclase and activation of PLC.

Several studies have shown that the D1 receptor agonist, eg. SKF 82526 induces stimulation of PLC activity in several tissues, including in striatal membranes, retinal horizontal and rat ovarian cells; the action of the D1 agonist on PI turnover can be blocked by the D1 antagonist, eg. SCH 23390, suggesting that this PI signal is mediated by a pharmacological dopamine-D1 receptor (Felder, et al 1989; 1990; Rodrigues et al, 1990; Undie et al, 1990). Despite effort, the cloning of the D1-like receptor which strongly couples to PLC has been unsuccessful to date (Sibley et al, 1992; Civelli et al 1993), although it is still possible that some D1-like receptors which couple to PLC remain to be identified. In our studies, it was found that the cloned human D1 receptor can induce a weak PI signal in Ltk- cells and this PI signal is subject to amplification by PKA activation. From present studies, we do not know the relationship between the cloned human D1 receptor and the receptor which mediated the D1 agonist-induced PI turnover in kidney cells or in striatum (Sibley et al, 1992). However, it is plausible to believe that this D1-like receptor, like the cloned human D1 receptors induce cell-specific activation of PLC.

The G protein involved in Gs-coupled receptor-mediated stimulation of PLC remains to be further elucidated. In COS-7 cells, PTH receptor-mediated increase of IP3 is a GTP-dependent response, suggesting mediation by a G protein. CTX has been reported to stimulate PI turnover in cloned pituitary cells and smooth muscle cells (Lo & Hughes, 1987; Xuan et al

1987). In our experiments, CTX pretreatment blocked the D1 receptor-induced increase in [Ca++]i in Ltk- cells, suggesting that Gs or a Gs-like protein may be involved in this response. To date, there is no evidence for a new CTX substrate. In the light of recent evidence that ßγ dimers can directly stimulate PLC-ß *in vitro* as well as *in vivo* (Camps et al, 1992: Katz et al, 1992), it is possible that the D1 receptors induce stimulation of PLC by releasing certain combinations of ßγ dimers.

An interesting question is the physiological significance of the receptor-mediated stimulation of PLC activity in Ltk- cells. It is clear that in GH4C1 cells, stimulation of adenylyl cyclase by VIP induced the release of growth hormone and prolactin while inhibition of cAMP synthesis and calcium entry blocked the hormone release (Dorflinger & Schonbrunn 1983; Enyeart et al 1990). From the present studies, we do not know the physiological functions of this signal in Ltk- cells. However, there is also no direct evidence for physiological significance of dopamine or 5-HT receptor-mediated inhibition or stimulation of adenylyl cyclase activity in the CNS. The present studies demonstrate that when different cellular milieu is provided, G protein coupled receptors may mediate different signal transduction pathways. Perhaps, in different cell systems, a given G protein coupled receptor exert different physiological function by producing different second messages.

It is noteworthy that Gi/Go coupled receptors, eg. 5-HT1A and D2 receptors inhibited basal activity in GH4C1 cells, but did not alter the basal adenylyl cyclase activity in Ltk- cells. These results indicate that the mechanisms of regulation of adenylyl cyclase activity in GH4C1 cells are different from the Ltk- cells. It has been suggested, although no actual data have been presented, that in peripheral tissues, where adenylyl cyclase subtypes (types I, III) are insensitive

to ßγ dimers, α subunits may mediate hormonal effects and ßγ dimers recycle α subunits for renewed activation. While in the CNS, some endocrine, exocrine, and epithelial cells, signalling is mediated by α subunit at low receptor occupancy and ßγ dimer at high receptor occupancy (Birnbaumer 1992). Our results first show the basic differences of Gi/Go-mediated regulation of adenylyl cyclase activity in GH4C1 and Ltk- cells: Ltk- cells lack Gi/Go-regulated basal level of adenylyl cyclase activity.

II. Modulation of Receptor Signalling by PKA and PKC

Exposure of receptors in intact cells or tissues to agonist leads to desensitization (Lefkowitz et al, 1990). Protein kinase A or C are involved in heterologous desensitization, a process whereby activation of one type of receptor causes desensitization of other types of receptors as well. It has been reported that uncoupling of receptor with G proteins is one of the biochemical changes during desensitization, and occurs more rapidly than other biochemical changes, eg receptor sequestration, internalization, etc. Activation of PKC has been reported to serve as a negative feedback pathway to inhibit receptor-mediated stimulation of PLC. In Swiss 3T3 fibroblast cells, prior activation of PKC by TPA inhibited bradykinin-mediated PI turnover (Brown et al, 1987). In olfactory epithelium cells, PKC selectively inactivated the odorant receptor-induced increase in IP3 while having no effect on odorant receptor-mediated cAMP signal (Boekhoff & Breer, 1992). In smooth muscle cells, activation of PKC by TPA inhibited angiotensin-mediated increase in PI turnover (Brock et al, 1986). These studies indicate that activation of PKC limits the PI signal induced by Gq-coupled receptors. A large body of evidence suggests that phosphorylation of hormone receptors is at least in part involved in this uncoupling (Lefkowitz et al 1990).

In our experiments, TPA inhibited the increase in PI turnover and [Ca++]i by the Gi/Go coupled (5-HT1A D2S, D2L) and the Gs-coupled receptor (D1). We also observed that TPA inhibited thrombin and ATP-induced increase in [Ca++]i which are partially or wholly mediated by Gq in Ltk- cells. These observations suggested that activation of PKC by TPA, or DAG, the endogenous activator for PKC, may be a common pathway to turn off PI signal induced by any G protein coupled receptor in L cells. It is unclear at present which signalling component is affected by PKC. One study showed that PKC can induce phosphorylation of the transfected human 5-HT1A receptors (Raymond 1991). To date, there is no evidence for phosphorylation of the D2 receptor by PKC. It is possible that phosphorylation of the receptor may account in part for the inhibitory action of TPA; but it is unclear why phosphorylation of the 5-HT1A receptor only uncouples receptor-induced PLC pathway without affecting inhibition of cAMP accumulation pathway.

Activation of PKA by 8-Br-cAMP did not directly alter activation of PLC and increase in [Ca++]i elicited by the 5-HT1A, D2S, or D2L receptors. These results are consistent with previous report that prior activation of PKA does not alter the D2S receptor-induced inhibition of adenylyl cyclase in Ltk- cells (Bates et al, 1990). From present studies, we do not know the biochemical mechanisms of potentiation of the inhibitory action of PKC by PKA in Ltk- cells. One study showed that activation of PKA inhibits DAG release in NCB-20 cells (Broke et al 1991). Because PKA has no direct effect on receptor-mediated PLC activation, the action of PKA on DAG release in Ltk- cells may only be detected when PKC is pre-activated.

The potentiation of the D1-receptor-mediated PI turnover by PKA was a surprising result. It is well documented that PKA induces phosphorylation of the ß-adrenergic receptor and causes

7uncoupling of the receptor with Gs and inhibits the receptor-mediated stimulation of adenylyl cyclase (Bouvier et al 1990; Lohse et al 1991). This appears to be the case in the D1 receptor-mediated stimulation of adenylyl cyclase as well. It has been shown that PKA activation reduced the affinity of the D1 receptor to agonist and inhibited the receptor-mediated inhibition of adenylyl cyclase (Machida et al, 1992). Our preliminary data also showed that pretreatment with 8-Br-cAMP inhibited the D1-receptor-mediated stimulation of cAMP accumulation in Ltk- cells. Considering that prior activation of PKA rather potentiated the action of the D1 receptor-mediated PI signal, while feedback inhibiting the cAMP signal, it would be expected that the PI signal might become the major signal for the D1 receptor in Ltk- cells while the cAMP signal is desensitized.

Taken together, activation of PKC inactivated the 5-HT1A, D2 receptor-mediated PI signal but had no effect on the receptor-mediated inhibition of adenylyl cyclase; while activation of PKA inhibited the D1-receptor-mediated stimulation of adenylyl cyclase but potentiated the receptor-mediated increase of PI turnover. Thus, prior activation of PKA or PKC in intact cells may not turn off all signals induced by hormone receptors, but rather selectively modulates different signal transduction pathways.

III. The Specificity of Receptor Coupling to Distinct G Proteins

The specific interaction of a given G protein coupled receptor with distinct G proteins has been the subject of intensive investigation using a variety of experimental approaches. It has become more and more clear that a given G protein coupled receptor can interact with more than one G protein. For example, both Gi2 and Gi3 were reported to be involved in the α2-C10 adrenergic receptor-mediated activation of PLC in rat-1 fibroblast cells (Milligan et al, 1992).

Of interest is PTX-sensitive G protein coupled receptors, which may interact with more than one G proteins to mediate multiple signal transduction pathways. Several lines of evidence have indicated that at least in neurons and in endocrine cells, Go specifically triggers closure of calcium channels whereas Gi3 selectively links opening of potassium channels (Kleuss et al , 1990; Yatani et al 1988). Consequently, a given Gi/Go coupled receptor couple to distinct G proteins to mediate activation or inhibition of different channels. For example, the dopamine-D2 receptor couples to Go to inhibit calcium currents and links to Gi3 for opening of potassium channels in (LIedo et al, 1991).

PTX-sensitive G protein coupled receptor-mediated inhibition of adenylyl cyclase is extremely complex. It has been postulated that the mechanisms of regulation of adenylyl cyclase in peripheral tissues are different from those in neurons and endocrine cells (Birnbaumer, 1992). GH4C1 pituitary and Ltk- fibroblast cells are the two typical examples to represent cell-specific mechanisms of regulation of adenylyl cyclase activity by Gi proteins. In GH4C1 cells, Gi proteins mediate both basal and Gs/forskolin-stimulated adenylyl cyclase, while in Ltk- fibroblast cells, Gi only involve in Gs/forskolin-stimulated adenylyl cyclase. A living cell system express multiple hormone receptors and G proteins. Mutation analysis has indicated that any of αi subunits may be capable to inhibit cAMP production, suggesting that any of Gi-coupled receptor can inhibit adenylyl cylcase activity (Wang et al 1990). It remains unclear whether a given Gi/Go coupled receptor interacts with one Gi/adenylyl cyclase complex or multiple Gi.adenylyl cyclase cyclase complexes.

Introduction of antisense mRNA into the living cells by transfection of antisense constructs to deplete gene expression has been utilized as a tool to study function of any protein

in mammalian system. This approach is limited because depletion of gene expression is often incomplete, and the specificity is uncertain. In the present experiment, hygromycin B plasmid, PY-3 was co-introduced with G protein αi.αo antisense constructs into GH4C1 cells. Hygromycin B is a cytotoxic reagent, which, at higher concentration, kills the cells with the low level of expression of PY-3 mRNA. In order to achieve the cell clone with highest expression of antisense mRNA and complete ablation of the desired G protein α subunit, the stable transfection procedure was modified: ten-time higher concentration of plasmid was used for transfection, and the selection procedure was divided into two steps, 1) to kill the cells without PY-3 plasmid and allow the cells to adapt toxicity of hygromycin B, 2) to select the aclones with the highest expression of α subunit antisense using higher concentration of hygromycin B. Using this modified stable transfection method, it was possible to specifically and significantly eliminate distinct G protein α subunits from a mammalian cells. The resulting antisense transfectants provided the excellent cell models for dissecting the functions of the α subunit in coupling of receptors to multiple effectors.

Using different approaches, including reconstitution experiments, introduction of antibody or antisense oligonucleotides to the cells (Kleuss et al, 1990; Lledo et al, 1991; McFadzean et al, 1989), it has been found that αo is involved in hormone receptor-mediated inhibition of calcium currents in GH3 pituitary tumour cells and NG108-15 neuroblastoma x glioma hybrid cells. Our results are consistent with these studies. It was found that elimination αo specifically abolished inhibition of the BAY K8644-induced increase in [Ca++]i induced by several Gi/Go coupled receptors, eg. somatostatin, muscarinic-M4, dopamine-D2S and D2L receptors, suggesting that all receptors examined triggers closure of voltage-dependent calcium channels

via Go.

The antisense transfectants also allowed us to simultaneously determine the role of αo in mediation of several effector activities, eg. adenylyl cyclase, potassium channels. It was observed that in αo eliminated clones, both basal and VIP-stimulated cAMP accumulation remained the same as the control cells. These results demonstrate that αo subunits are not involved in the regulation of adenylyl cyclase activity in any manner in GH4C1 cells.

In GH4C1 cells, stimulation of dopamine-D2S, D2L, somatostatin, muscarinic-M4 receptors inhibited the BAY K8644-induce increase in [Ca++]i. In αo-depleted clones, the inhibitory actions of D2S, somatostatin and muscarinic-M4 receptors on [Ca++]i were completely abolished; however, the action of D2L receptor on [Ca++]i was retained by 30%. This partial inhibitory action on calcium entry induced by the D2L receptors was blocked by PTX pretreatment, suggesting the mediation by Gi proteins. The involvement of Gi protein in inhibition of calcium entry remains controversial. While Go has been found to couple to several receptors to inhibit voltage-dependent calcium channels in heart, neuroblastoma, endocrine cells, it is generally believed that Gαi may be able to couple to calcium channels as well but are less effective than Gαo (Birnbaumer et al, 1990). However, it is unclear when Gi proteins may be involved in inhibition of calcium entry. The present studies first indicate the conditional replacement of αo by αi to mediate inhibition of calcium entry in GH4C1 cells provided that αo is depleted in GH4C1 cells. This result is particularly interesting. As has been discussed in Chapter II, the genes encoding G proteins are housekeeping genes, their expression are subjected to the regulation by variety of compounds, eg, hormone, receptor antagonists etc. It is possible that sometimes the amount of αo in certain types cells may be significantly reduced due to drug

treatment (receptor agonist or antagonist). Under this condition, some hormone receptors may couple to Gi proteins to inhibit voltage-dependent calcium channels as an accessory signal transduction pathway.

Mutagenesis has indicated that any of αi subunits can inhibit adenylyl cyclase (Wang et al 1992). However, no direct evidence is available for the linkage of receptor and adenylyl cyclase in intact cells. As discussed above, for neurons and endocrine cells, there are more than one mechanisms for inhibition of adenylyl cyclase by Gi, eg inhibition of basal adenylyl cyclase activity and inhibition of Gs- or forskolin-stimulated adenylyl cyclase activity. An important question is which subunit regulates basal adenylyl cyclase activity and which regulates Gs-stimulated adenylyl cyclase activity.

In the present studies, it was found that inhibition of basal cAMP accumulation by the dopamine-D2S, D2L, somatostatin and muscarinic-M4 receptors was blocked in any of αi2-antisense clones, suggesting that αi2 is crucial for receptor-mediated inhibition of basal adenylyl cyclase activity in GH4C1 cells. The elimination of αi2 subunits had a clearly different effect on receptor-mediated inhibition of VIP-stimulated cAMP accumulation. The action of the D2S and muscarinic-M4 receptors was blocked, suggesting that αi2 is essential for these two receptors to inhibit both basal and Gs-stimulated adenylyl cyclase activity in GH4C1 cells. The action of the D2L receptor on VIP-stimulated adenylyl cyclase activity remain almost unaltered, suggesting that the D2L receptors couple to Gi2 to inhibit basal adenylyl cyclase, while linking Gi1/Gi3 to regulate VIP-stimulated adenylyl cyclase activity. Apparently, some hormone receptors may link to different αi subunits to regulate different levels of adenylyl cyclase activity in mammalian cell system. Consistently, it was also found that in αi1-depleted GH4C1 cells,

the 5-HT1A receptors stimulated basal cAMP accumulation but inhibited VIP-stimulated cAMP accumulation; whereas in αi3 eliminated cells, the dopamine-D2S receptors increased basal cAMP accumulation while inhibiting the action of VIP (Liu & Albert, unpublished observations). These results further reveal that hormone receptors regulate different levels of adenylyl cyclase via distinct Gi proteins.

On the other hand, somatostatin receptors potentiated the action of VIP by 30%. The reason for the potentiation of the action of VIP by somatostatin receptor but not the D2 or M4 receptors is not addressed here. Based on the studies that somatostatin receptors in GH3 cells may select certain βγ dimers (β1γ3) to mediate calcium channels (Kleuss et al, 1992; 1993); and that free βγ dimers released from Gi can stimulate adenylyl cyclase types II and IV (Tang & Gilman, 1991; Federman et al, 1992), one hypothesis is that different hormone receptors can select different βγ dimers, while only certain type of βγ dimer combinations can stimulate adenylyl cyclase type II found in GH4C1 cells; since different receptor may release different βγ dimers upon activation, only certain receptors can potentiate Gs-stimulated adenylyl cyclase activity. Further experiments are needed to verified this hypothesis.

The difference of transmembrane signalling of the short and long forms of the dopamine receptors has been drawn good attention. Despite a good body of evidence for involvement of the third cytoplasmic loop in G protein signalling where these two forms of the D2 receptors differ, no evidence for a difference in signal transduction for the two forms of the D2 receptors has been reported. From our characterization studies, it was observed that the D2S and D2L receptors exhibit similar signalling pattern in both GH4C1 and Ltk- cells. However, it was found that the two D2 receptors exhibit clear different sensitivity to PKC, suggesting the

presence of differences in molecular interaction with intracellular proteins for these two D2 receptors. Indeed, it was observed that clear differences in coupling to Go and Gi2 for D2S and D2L receptors exist.

For receptor/G protein signalling, an important question is the specificity of G protein coupling to different receptors. From the present studies, it is clear that the receptors examined can couple to every G protein α subunit in GH4C1 cells, and each of them triggers the specific signal, eg. αo triggers inhibition of closure of calcium channels, while αi2 initiates inhibition of adenylyl cyclase. For adenylyl cyclase activity, different receptors interact with different αi subunit to regulate different levels of the enzyme activity. The picture here is that although these receptors interact with the same Gα, they are different in coupling to effectors.

In GH3 cells, the presence of all four ß and five γ subunits has been reported (Kleuss et al, 1992; 1993). Theoretically, it will have 20 isoforms of Go1, Go2, Gi1, Gi2 or Gi3. Since hormone receptors can select ßγ dimers to mediate voltage-dependent calcium channels, they may be also able to select ßγ dimers to inhibit adenylyl cyclase. Perhaps, different levels of adenylyl cyclase are regulated by different isoforms of Gi proteins, while different receptors couple to distinct isoforms of G proteins to regulate different subtypes of effectors.

Taken together, the present studies demonstrate that G protein α subunits determine the signalling phenotypes (eg. closure of calcium channels, inhibition or stimulation of adenylyl cyclase or PLC etc) as well as the specificity of coupling to certain types of effectors; while ßγ dimers mainly determine the specificity of G protein signalling. In other word, different combinations of Gα and Gßγ dictate the foundation of the specific interaction of receptors and effectors.

Table 7.1. The signals induced by the 5-HT1A, dopamine-D1, -D2S and D2L receptors in GH4C1 pituitary and Ltk- fibroblast cells.

Transfected	GH4C1 cells		Ltk- cells	
Receptors	AC[1]	Ca++ Channel	AC[2]	PLC
r5-HT1A	↓	↓	↓	↑↑
hD2S	↓↓	↓↓	↓↓	↑↑↑
hD2L	↓↓	↓↓	↓↓	↑↑↑
hD1	↑↑	↑	↑↑	↑

Abbreviations: AC, adenylyl cyclase; PLC, phospholipase C, Ca++ channel, voltage-dependent calcium channels.

Note: 1. Inhibition of AC activity by the receptors includes both basal and VIP stimulated AC activity;

2. Inhibition of AC activity by the receptors refers as forskolin-stimulated AC activity.

Table 7.2. The effects of 8-Bro-cAMP and TPA on the dopamine-D1, D2S, D2L and 5-HT1A receptor-induced cAMP and PI signals.

	PKA		PKC	
	cAMP	PI	cAMP	PI
Dopamine-D1	↓	↑↑	-	↓
Dopamine-D2S	-	↓(By PKC)	-	↓↓
Dopamine-D2L	ND	↓(By PKC)	-	↓
5-HT1A	ND	↓(By PKC)	-	↓↓

Note: "-", no effect; ND, not determined; ↓, inhibition; ↑↑, potentiation, ↓(By PKC), potentiates the inhibitory action of PKC.

Table 7.3. The nomenclature of αi/αo-antisense transfectant cells.

Antisense	r5-HT1A	rD2S	hD2L
Control	GH4ZD10	GH4ZR7	GH4D2L
anti-αo	none	GoZR7	GoD2L
anti-αi2	Gi2ZD10	Gi2ZR7	Gi2D2L

Table 7.4. Summary of elimination of αo or αi2 on receptor-induced inhibition of cAMP accumulation and calcium influx.

	αo			αi2		
	BcAMP	VcAMP	Ca++	BcAMP	VcAMP	Ca++
D2S	-	-	↓↓	↓↓	-	-
D2L	-	-	↓ (70%)	↓↓	↓↓	-
SS	-	-	↓↓	↓↓	↑ (30%)	-
M4	-	-	↓↓	↓↓	↓↓	-

Note: "-", no effect; "↓↓", completely blocks; ↓(70%), blocks by 70%; ↑(30%), enhances by 30%; SS, somatostatin; BcAMP, basal cAMP accumulation; VcAMP, VIP-stimulated cAMP accumulation.

CHAPTER VIII SUMMARY OF CONTRIBUTIONS TO THE LITERATURE

CHAPTER VIII SUMMARY OF CONTRIBUTIONS TO THE LITERATURE

1. Cell-specific signalling of G protein coupled receptors

Using two distinct cell systems, GH4C1 pituitary and Ltk- fibroblast cells, as models, the signal transduction pathways of several G protein-coupled receptors, including 5-HT1A, dopamine-D2S, -D2L, and D1 receptors were characterized and the cell-specificity of Gi/Go- or Gs-coupled receptor signalling was clearly revealed in the present studies. In GH4C1 cells, G protein coupled receptors exhibit similar signalling phenotypes as found in neurons, while in Ltk- fibroblast cells, the signalling phenotypes of these G protein coupled-receptors are similar to those observed in peripheral cell systems. More importantly, GH4C1 pituitary and Ltk- fibroblast cells may represent two major classes of G protein signalling systems: neuronal signalling system and peripheral signalling system.

2. Alteration of receptor signalling by protein kinases A and C

Protein kinases A and C are known to be involved in both homologous and heterologous desensitization of G protein-coupled receptors (Lefkowitz et al 1990; Dolhman et al, 1991; Kobilka 1992). However, in present studies, it was found that prior activation of protein kinase A or C did not turn off all signals elicited by the receptors: some signals were inhibited, some remained unaltered, some were even enhanced upon activation of protein kinase. Thus activation of protein kinases A and C does not simply desensitize receptor signalling, but rather alter its signal transduction pathways. This finding indicates that protein kinases A and C are the regulatory components for transmembrane signalling and cross-talk of G protein-coupled receptors.

3. Different sensitivities of D2S and D2L receptor signalling to protein kinase C in Ltk- cells It was found that prior activation of protein kinase C can completely block the PI signal induced by the D2S receptors but not the PI signal mediated by the D2L receptors in Ltk- fibroblast cells. This results suggest that protein kinase may selectively modulate the function of the two forms of two D2 receptors.

4. Modification of stable transfection procedure

Transfection of antisense constructs into mammalian cells to eliminate the gene expression of a desired gene is a very powerful tool to study function of any proteins. However, this tool has been limited because depletion of gene expression is incomplete and the specificity is uncertain. We have modified stable transfection procedure. This procedure was utilized to stably and separately transfect distinct antisense constructs of α subunits into GH4C1 cells; and the antisense transfectant clones with specific and complete elimination of distinct α subunits were achieved. This procedure may be useful to eliminate the gene expression of other proteins in mammalian system and to study their functions in intact cells.

5. Regulation of adenylyl cyclase activity by G proteins is a dynamic process

By simultaneously dissecting G protein signalling of several hormone receptors, including dopamine-D2S, -D2L, muscarinic-M4, somatostatin receptors, it was found that different receptors may link to different G proteins to regulate basal and VIP-stimulated cAMP synthesis. It was also observed that the coupling of receptor to Gi proteins was subject to alter upon a Gs-coupled receptor activation.

6. The specificity of G protein signalling

By elimination of distinct G protein α subunits from GH4C1 pituitary cells, it was found that αo selectively linked receptor-mediated closure of voltage-dependent calcium channels but not inhibition of adenylyl cyclase, whereas $\alpha i2$ were coupled to inhibition of adenylyl cyclase but not closure of calcium channels. Both α subunit and $\beta\gamma$ dimer are all involved in the specificity of G protein signalling. The variety of G protein isoforms from different combinations of α subunits and $\beta\gamma$ dimers determine the specificity of receptor coupling to G proteins.

7. Differences of dopamine-D2S and -D2L receptors in coupling to Go and Gi2

The long and short forms of dopamine-D2 receptors differ at the third cytoplasmic loop where the long form of D2 receptor has a 29 amino acid insert (Civelli et al 1993). Since the third cytoplasmic loop is known to be involved in coupling to G proteins (Dohlman et al 1991), it is plausible to believe that these two forms of dopamine-D2 receptor may differ in coupling to G proteins. Despite much effort, no evidence for the difference of the D2S or D2L receptor signalling has been presented (Civelli et al 1993). Since PTX-sensitive G protein share considerable amino acid sequence identity and certain, if not all, functions, it is difficult to illustrate the difference in coupling to distinct G proteins for these two D2 receptors by characterization of their signal transduction pathways.

By elimination of αo or $\alpha i2$ subunits from GH4C1 cells, the different specificity of dopamine-D2S and D2L receptors in coupling to Go and Gi2 were revealed: the D2S receptors mediated inhibition of calcium entry solely via Go whereas the D2L receptor can recruit Gi

proteins to mediate this signal when αo subunits were depleted; the D2L receptor, via Gi2, inhibited both basal and VIP-stimulated cAMP synthesis, while αi2 was only involved in the D2S receptor-mediated inhibition of basal adenylyl cyclase activity.

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APPENDIX A PUBLICATIONS WITH DR. R, QUIRION

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Presence of Various Carbohydrate Moieties Including β-Galactose and *N*-Acetylglucosamine Residues on Solubilized Porcine Brain Neurokinin-1/Substance P Receptors

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Abstract: Neurokinin-1 (NK-1)/substance P (SP) receptors were solubilized using 10 m*M* 3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfate from porcine striatal membranes (solubilization yield, 80%). In solubilized preparations, [^{3}H]SP apparently bound to a single class of high-affinity sites (K_D = 0.82 ± 0.13 n*M*) as in membrane homogenates. The ligand selectivity pattern observed in both membrane and solubilized receptor preparations indicated that [Sar^9,Met(O$_2$)^{11}]SP = SP ≫ senktide = [Nle^{10}]neurokinin A$_{4-10}$. This suggests the selective labeling of the NK-1 receptor class in both assays. Solubilized receptors were retained on agarose-coupled lectins that bind *N*-acetylglucosamine-galactose and β-galactose (*Ricinus communis* I and *Ricinus communis* II), mannose (concanavalin A and lentil), and *N*-acetylglucosamine (wheat germ agglutinin) but not on lectins binding fucose (*Lotus* A) and *N*-acetylgalactosamine (*Dolichos biflorus* A). Thus, it appears that porcine brain NK-1/SP receptors are enriched with various carbohydrate moieties, β-galactose and *N*-acetylglucosamine-galactose residues being especially abundant. This situation is rather different from that in various other members of the rhodopsin seven-transmembrane receptor superfamily. **Key Words:** Neurokinin—Substance P—Carbohydrate—β-Galactose—Solubilization.
Liu Y. F. and Quirion R. Presence of various carbohydrate moieties including β-galactose and *N*-acetylglucosamine residues on solubilized porcine brain neurokinin-1/substance P receptors. *J. Neurochem.* 57, 1944–1950 (1991).

Cloning of membrane-bound receptors provides a powerful tool for understanding the organizational feature of neurotransmitter receptors. Recently, many guanine nucleotide regulatory protein (G protein)-coupled receptors such as the β_1 (Frielle et al., 1987) and β_2 (Dixon et al., 1986) adrenergics, neurokinin-2 (NK-2) or substance K (Masu et al., 1987), serotonin$_{1A}$ (5-HT$_{1A}$) (Albert et al., 1990), and various muscarinic cholinergic (Bonner et al., 1987; Peralta et al., 1987; Bonner, 1989) receptors have been cloned (for recent reviews, see Bonner, 1989; O'Dowd et al., 1989). Significant sequence homologies are found among all these receptors, translating into a conserved seven-transmembrane-spanning three-dimensional structure (Bonner, 1989; O'Dowd et al., 1989). Another common structural feature of these receptors is related to the presence of potential N-linked glycosylation sites at the amino terminal (O'Dowd et al., 1989; Kobilka, 1990; Rands et al., 1990).

Glycosylation has been demonstrated to be essential to ensure the proper function and subcellular distribution of several surface glycoproteins. However, the precise role of glycosylation sites in membrane-bound receptors is still unknown. Data derived from the mutation of N-linked glycosylation sites in β_2-adrenergic receptors suggested that those sites may be related to receptor stability and turnover from the membrane pool (Kobilka, 1990; Rands et al., 1990). They do not appear to be essential for ligand binding affinity and receptor activation (Kobilka, 1990; Rands et al., 1990).

Neurokinin-1 (NK-1)/substance P (SP) receptors are widely distributed in mammalian tissues including brains (Quirion et al., 1983; Shults et al., 1984; Beaujouan et al., 1986; Quirion and Dam, 1988). Recently,

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Abbreviations used: Asn, asparagine; CHAPS, 3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate; G protein, guanine nucleotide regulatory protein; Gpp(NH)p, guanylylimidodiphosphate; NK, neurokinin; PEG, polyethylene glycol 8000; PMSF, phenylmethylsulfonyl fluoride; SP, substance P; WGA, wheat germ agglutinin.

this receptor class has been cloned (Yokota et al., 1989; Hershey and Krause, 1990). As for the NK-2 (Masu et al., 1987) and NK-3 (Shigemoto et al., 1990) receptors, it belongs to the G protein-coupled seven-transmembrane receptor superfamily. In agreement with previous biochemical studies (Maruyama, 1986; Dam et al., 1987; Nakata et al., 1988*a,b*), the cloned NK-1 receptor has an apparent molecular mass of 43–46 kDa and possesses potential N-linked glycosylation sites (Asn^{14} and Asn^{18}) (Yokota et al., 1989; Hershey and Krause, 1990). However, the nature of the putative carbohydrate moieties present on the NK-1 receptor is unknown. This was investigated in the present study using porcine striatal 3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized membrane preparations and lectin–agarose affinity chromatography, which can effectively fractionate Asn-linked carbohydrate chains (for reviews, see Hedo, 1984; Goldstein and Hayes, 1978). CHAPS-solubilized NK-1 receptors were retained mostly by columns immobilized with *Ricinus communis* I and II, concanavalin A, and wheat germ agglutinin (WGA). *Dolichos bioflorus* A and *Lotus* A agarose-coupled lectins did not retain solubilized NK-1 sites. This indicates that the NK-1 receptor is a glycoprotein enriched with various carbohydrate moieties including β-galactose, *N*-acetylglucosamine–galactose, mannose, and *N*-acetylglucosamine-type residues.

MATERIALS AND METHODS

Materials

[^{3}H]SP (36–40 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). SP, [Sar^9,$Met(O_2)^{11}$]SP, senktide, and [Nle^{10}]NK A_{4-10} were obtained from IAF Biochemical Inc. (Laval, Quebec, Canada). Lectins coupled to agarose beads and the appropriate sugar haptens were from E.Y. Laboratories (San Mateo, CA, U.S.A.). CHAPS was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.), while polyethylene glycol 8000 (PEG) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Salts, protease inhibitors, γ-globulin, and other chemicals of general use were purchased from either Sigma Chemical Co. or Fisher Canada Ltd. (Montreal, Quebec, Canada). Whatman GF/B filters and scintillation cocktail were obtained from Fisher Canada Ltd. Porcine brains were obtained fresh from a local slaughterhouse (R. Boucher, St. Jean Baptiste de Rouville, Quebec, Canada).

Membrane preparation

Fresh pig brains were dissected on ice and striatal membranes were prepared as follows: porcine striata were homogenized (Brickman Polytron at setting 6) in 50 m*M* ice-cold Tris·HCl (pH 7.4) buffer containing 150 m*M* NaCl, 150 m*M* KCl, 12 m*M* EDTA, 200 μ*M* phenylmethylsulfonyl fluoride (PMSF), 40 μg/ml bacitracin, 4 μg/ml leupeptin, and 2 μg/ml chymostatin. These homogenates were then incubated for 30 min at 4°C before being centrifuged at 30,000 *g* for 20 min at 4°C and washed twice with 50 m*M* Tris·HCl (pH 7.4) buffer. Pellets were resuspended in 0.32 *M* sucrose containing 200 μ*M* PMSF and 40 μg/ml bacitracin before storage at −80°C until use.

Binding assay in membrane homogenates

Sixty-minute incubations were carried out at room temperature in 50 m*M* Tris·HCl (pH 7.4) buffer containing various concentrations of [^{3}H]SP (0.05–20 n*M*), 5 m*M* $MgSO_4$, 40 μg/ml bacitracin, 4 μg/ml leupeptin, and 2 μg/ml chymostatin (Dam et al., 1987) in the presence of 0.8–1 mg of membrane protein (Bradford, 1976) in a final volume of 1 ml. Total binding and nonspecific binding were determined in triplicate in the absence or presence of 1 μ*M* unlabeled SP. Incubations were terminated by adding 4 ml of ice-cold 50 m*M* Tris·HCl (pH 7.4) buffer and membranes were filtered (Cell Harvester; Brandel Instruments, Gaithersburg, MD, U.S.A.) on Whatman GF/B glass-fiber filters that had been presoaked in 0.5% polyethylenimine for a minimum of 3 h to reduce absorption. Filters were then washed three times (5 ml each) using ice-cold Tris·HCl (pH 7.4) buffer and bound radioactivities were determined using a Beckman liquid scintillation counter with a 45% efficacy. Under these assay conditions, specific [^{3}H]SP binding represented between 75 and 80% of the total binding at low-nanomolar concentrations. Saturation and competition data were analyzed using a computerized nonlinear least-squares curve-fitting technique (Bio-Soft, Elsevier, England).

Solubilization of [^{3}H]SP binding sites

Membrane preparations were centrifuged as above at 30,000 *g* for 20 min at 4°C to remove sucrose, and pellets resuspended in 50 m*M* Tris·HCl (pH 7.4) buffer containing 10 m*M* CHAPS, (Nakata et al., 1988*a,b*; Morishima et al., 1989), 1 *M* NaCl (Maruyama, 1986), and 100 μ*M* PMSF (Gioannini et al., 1982), to yield a protein concentration (Bradford, 1976) of 3 mg/ml. This mixture was then slowly shaken in an ice bath for 1 h before centrifugation at 100,000 *g* for 1 h. Pellets were discarded, while supernatants were used as the source of solubilized [^{3}H]SP receptor protein.

Binding assay in CHAPS-solubilized fractions

Solubilized membrane preparations were mixed 1:1 in 40% PEG and centrifuged at 30,000 *g* for 20 min. This approach maximized the recovery of soluble receptor protein and improved the signal-to-noise ratio. Resulting pellets were resuspended in 50 m*M* Tris·HCl (pH 7.4) buffer. Receptor binding assays (approximately 0.3 mg protein) were then carried out for 60 min at room temperature in a 50 m*M* Tris·HCl (pH 7.4) buffer containing various concentrations of [^{3}H]SP (0.05–20 n*M*), 5 m*M* $MgSO_4$, 2 μg/ml chymostatin, 40 μg/ml bacitracin, and 4 μg/ml leupeptin in a final volume of 1 ml. Total and nonspecific bindings were determined as in membrane homogenates. Incubations were terminated by the addition of 4 ml of ice-cold 15% PEG buffer containing 0.1 ml of 0.02% γ-globulin solution, mixed before filtration on Whatman GF/B glass-fiber filters presoaked in 0.5% polyethylenimine. Filters were then washed once using 5 ml of PEG (7.5%). Radioactivity was evaluated as described for membrane binding. Under such assay conditions, specific binding accounted for about 80% of the total binding at concentrations close to K_D values.

Lectin chromatography

Lectin–agarose gels (10 ml) were washed using at least 200 ml of 50 m*M* Tris·HCl (pH 7.4) buffer containing 250 m*M* NaCl, 3 m*M* CHAPS, and 200 μ*M* PMSF (buffer A). Additionally, 1 m*M* $CaCl_2$ and 1 m*M* $MnCl_2$ were added for the concanavalin A–agarose-coupled lectin. The solubilized receptor preparation, diluted one- to threefold in buffer A, was applied to various lectin columns, and the flow was stopped

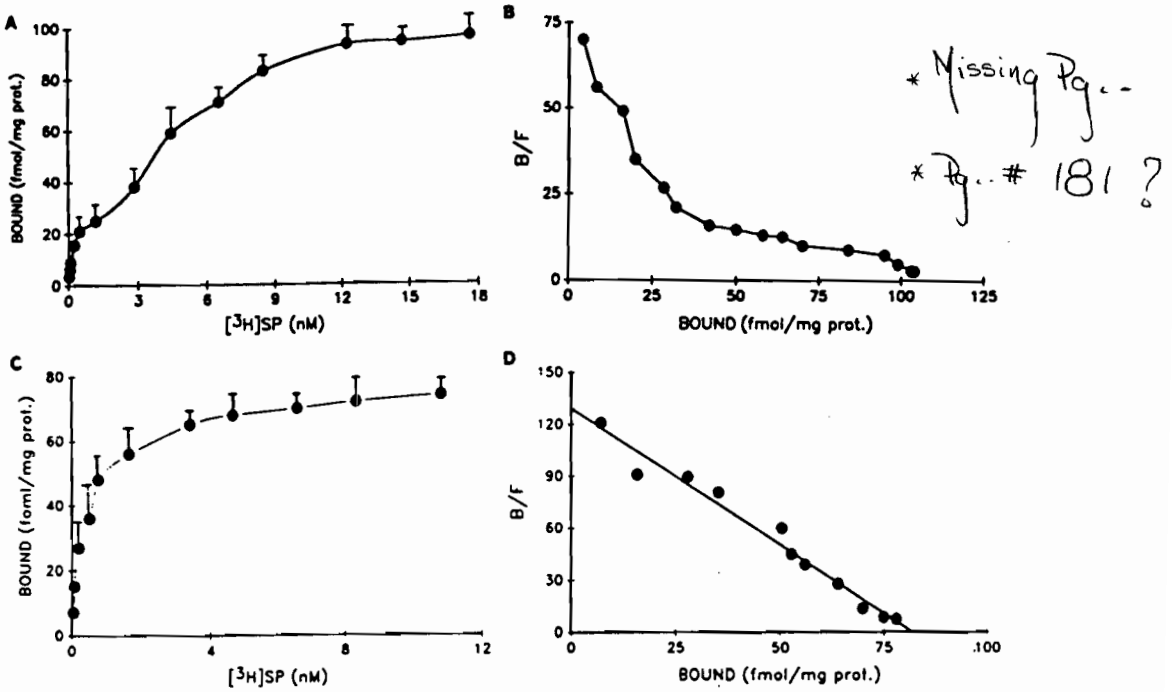

FIG. 2. Saturation curves (A, C) and Scatchard representations (B, D) of [^{3}H]SP binding to porcine striatal membrane homogenates (A, B) or CHAPS-solubilized fractions (C, D). Membrane homogenates and solubilized preparations were incubated in the presence of various concentrations of [^{3}H]SP as described under Materials and Methods. Values represent the means ± SEM of three separate experiments. Note that while a curvilinear Scatchard plot is obtained in homogenates (B), this is not the case in CHAPS-solubilized fractions (D).

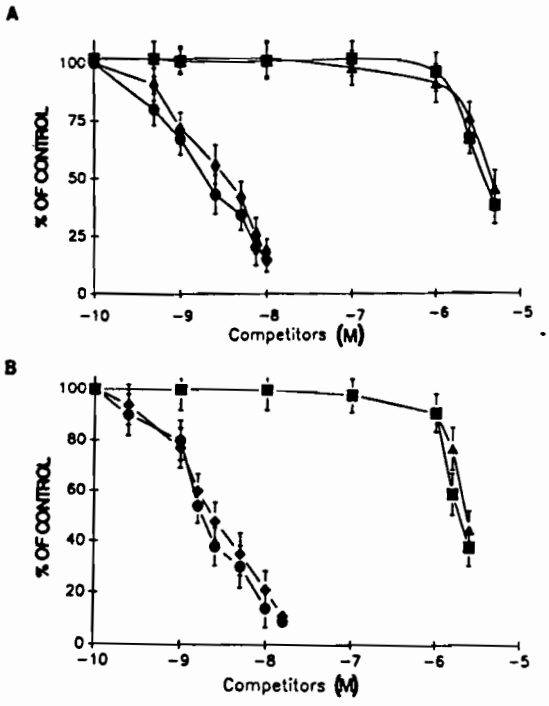

FIG. 3. Competition of [^{3}H]SP (1.0 n*M*) binding in membrane homogenates (A) and CHAPS-solubilized fractions (B) by various neurokinin peptides: ● —— ●, SP; ♦ —— ♦, [Sar^9,Met(O$_2$)^{11}]SP; ▲ —— ▲, [Nle^{10}]NK A$_{4-10}$; ■ —— ■, senktide. It is clear that only SP and the selective NK-1 agonist act as potent competitors in both assays. Values represent means ± SEM of at least three assays.

with IC_{50} values of 7.0 ± 1.0 and 5.0 ± 1.2 μM for GTP and Gpp(NH)p, respectively. This also suggests that a higher proportion of NK-1 binding sites is in the high-affinity G protein-coupled mode in CHAPS-solubilized preparations.

Carbohydrate composition of CHAPS-solubilized NK-1 receptors

The solubilized preparation was applied to various agarose-coupled lectin columns before elution. As shown in Table 1, [^{3}H]SP CHAPS-solubilized NK-1

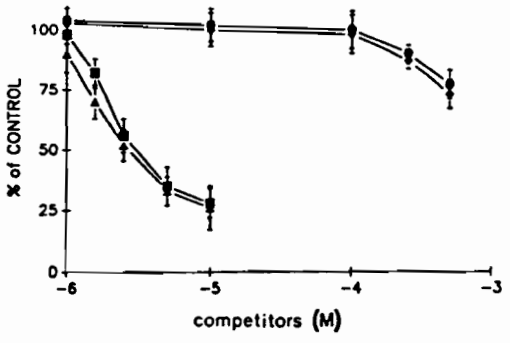

FIG. 4. Comparative effects of GTP and Gpp(NH)p on [^{3}H]SP (1.0 n*M*) binding to membrane homogenates and CHAPS-solubilized fractions. Gpp(NH)p [(▲ —— ▲) soluble and (● —— ●) membrane preparations] and GTP [(■ —— ■) soluble and (♦ —— ♦) membrane preparations] were apparently equally effective, being much more potent in solubilized preparations. Values represent means ± SEM of three to five different experiments.

TABLE 1. *Recovery of solubilized NK-1/SP receptors from various agarose-coupled lectins*

Lectin	Sugar eluant	Recovery of [^{3}H]SP binding (%)		
		Flow-through	Eluate 1	Eluate 2
Ricinus communis I	β-Methyl-D-galactopyranoside	40	35	11
Ricinus communis II	β-Methyl-D-galactopyranoside	45	28	12
Lentil lectin	α-Methyl-D-mannoside	75	11	<1
Concanavalin A	α-Methyl-D-mannoside	73	9	<1
WGA	*N*-Acetylglucosamine	56	20	7
Dolichos biflurous A	*N*-Acetylgalactosamine	94	<1	—
Lotus A	α-Fucose	95	<1	—

Values are means from at least three separate experiments. All fractions were ultrafiltered and concentrated using a Centricon 30 concentrator (Amicon) before [^{3}H]SP (1.0 n*M*) binding assay (see Materials and Methods for details).

receptor is not absorbed by the agarose-coupled lectins immobilized with *Dolichos biflorus* A and *Lotus* A. In contrast, *Ricinus communis* I and II are most efficient for retaining [^{3}H]SP/CHAPS-solubilized receptor proteins, indicating the existence of galactose and *N*-acetylglucosamine–galactose residues (Baenziger and Fiete, 1979; Hedo, 1984; Goldstein and Hayes, 1978) on the porcine striatal NK-1/SP receptor. This is also supported by the finding that about 40% of the total activity recovered is eluted by β-methyl-D-galactopyranoside (Table 1). Moreover, the β configuration of the D-galactoside sugar is most likely present since CHAPS-solubilized fractions cannot be retained by a lectin (*Dolichos biflorus* A) that binds other forms of the carbohydrate (Hedo, 1984; Goldstein and Hayes, 1978) (Table 1). Solubilized [^{3}H]SP receptor proteins are also retained, to some extent, by lentil, concanavalin A, and WGA lectin columns (Table 1), revealing the presence of α-mannose and *N*-acetylglycosamine, or sialic acid, moieties on NK-1 receptors (Hedo, 1984; Goldstein and Hayes, 1978; Cummings and Kornfeld, 1982; Kornfeld et al., 1981).

Up to 40–46% of bound [^{3}H]SP/NK-1 receptors are recovered from agarose-coupled lectins after two elutions (Table 1). This translated into 20-fold purification using the WGA column and up to 45-fold purification for the two *Ricinus communis* lectins (Table 2). Finally, we also observed that CHAPS-solubilized [^{3}H]SP/NK-1 receptors eluted from those columns are still fully sensitive to unlabeled SP and the selective NK-1 agonist, as well as to GTP and Gpp(NH)p (data not shown).

TABLE 2. *Purification and recovery of NK-1/SP receptors from* Ricinus communis *I and II and WGA agarose-coupled lectins*

	Recovery of [^{3}H]SP binding (%)	Purification (-fold)
WGA	27	21
Ricinus communis I	46	45
Ricinus communis II	40	42

Data are means of three separate experiments. Purification is calculated from the total specific binding of eluates (eluate 1 + eluate 2) normalized as femtomoles per milligram of protein divided by the total specific binding of the solubilized receptors (fmol/mg protein) applied to the column.

DISCUSSION

The most important finding of the present study relates to the demonstration that the recently cloned NK-1/SP receptor (Yokota et al., 1989; Hershey and Krause, 1990) is a glycoprotein enriched with various carbohydrate moieties, including β-galactose, *N*-acetylglucosamine–galactose complex, mannose, and sialic acid residues. This may have functional significance since it has recently been shown that mutation of glycosylation sites in the β_2-adrenergic receptor, another member of the rhodopsin receptor superfamily (O'Dowd et al., 1989), alters the process of insertion and distribution of this receptor on the cell surface (Kobilka, 1990) without directly affecting ligand specificity and affinity (Kobilka, 1990; Rands et al., 1990). However, this may not apply to all members of this receptor family since recent studies have demonstrated that sialic acid residues on M_2 muscarinic receptors (Herron and Schimerlik, 1983; Peterson et al., 1986) are directly involved in the formation of super-high-affinity agonist–receptor complexes (Gies and Landry, 1988: Haddad et al., 1990). Moreover, it has been shown that lectins such as concanavalin A can reduce desensitization of a neuronal type of quisqualate receptor (Mayer and Vyklicky, 1989). Thus, appropriate glycosylation may be critical to ensure maximal receptor affinity and response, different carbohydrate moieties on the receptor protein possibly modulating specific aspects of receptor pharmacology.

It is of interest that on the porcine striatal NK-1 receptor, β-galactose and *N*-acetylglucosamine–galactose residues appear to be abundant. This is unique since for most other G protein-coupled receptors, including M_2 muscarinic (Herron and Schimerlik, 1983; Peterson et al., 1986; Gies and Landry, 1988; Haddad et al., 1990), α- and β-adrenergic (Stiles et al., 1984; Cervantes-Oliver et al., 1985; George et al., 1986; Be-

novic et al., 1987; Regan, 1988), opiate (Gioannini et al., 1982), and neuropeptide Y (Sheikh and Williams, 1990) receptors, *N*-acetylglucosamine (or sialic acid) and mannose are most abundant, with *N*-acetylglucosamine–galactose and β-galactose residues rarely being reported. It is unclear if this is unique to NK-1 receptors and it would be of interest to determine if other NK receptors such as the NK-2 (Masu et al., 1987) and NK-3 (Shigemoto et al., 1990) sites are also enriched with such complex carbohydrate residues.

Other carbohydrate moieties also present on the NK-1 receptor include *N*-acetylglucosamine, α-mannose, and possibly hybrid-type carbohydrate chain since WGA retained the soluble protein (Yamamoto et al., 1981). However, fucose and *N*-acetylgalactosamine are probably absent or present in limited amounts (taking into consideration the limitation of methods used here), their corresponding agarose-coupled lectins (Kornfeld et al., 1981; Cummings and Kornfeld, 1982; Goldstein and Hayes, 1978; Hedo, 1984) failing to retain CHAPS-solubilized [^{3}H]SP binding proteins. Similar results have been reported for other members of the seven-transmembrane-segment receptor family, including M_2 muscarinic (Herron and Schimerlik, 1983; Peterson et al., 1986), α- and β-adrenergic (Stiles et al., 1984; Cervantes-Olivier et al., 1985; George et al., 1986; Benovic et al., 1987; Regan, 1988), opiate (Gioannini et al., 1982), and neuropeptide Y (Sheikh and Williams, 1990) receptors. Thus, the existence of N-linked glycoproteins, as already demonstrated for muscarinic (Bonner, 1989), β-adrenergic (Kobilka, 1990; Rands et al., 1990), and opiate (Gioannini et al., 1982) receptors, is also suggested for the NK-1 receptor and is consistent with recent data derived from the cloning of the mammalian brain NK-1/SP receptor (Yokota et al., 1989; Hershey and Krause, 1990).

As reported earlier using other tissues (Nakata et al., 1988*a,b*; Morishima et al., 1989) we observed that CHAPS is a most effective detergent for solubilizing NK-1/SP receptors present in porcine striatal membranes. The efficacy of CHAPS was markedly increased in the presence of a high concentration (1.0 *M*) of NaCl, as reported earlier for opiate receptors (Gioannini et al., 1982). The high yield (80%) observed in our study suggests that a mixture of CHAPS/NaCl is more efficacious than previously used detergents [such as glycodeoxycholate–NaCl (Maruyama, 1986)] to solubilize NK-1/SP receptors.

The ligand selectivity pattern observed in both membrane-bound and CHAPS-solubilized preparations reveals that [^{3}H]SP binds to the NK-1 receptor class (Quirion and Dam, 1988) since selective NK-1, but not NK-2 or NK-3, agonists were able to compete with nanomolar affinities for those sites.

In porcine striatal membrane preparations, it appears that NK-1 receptors exist in multiple (at least two) affinity states, with a large proportion of [^{3}H]SP binding exhibiting a low K_D value. In contrast, only a high-affinity [^{3}H]SP binding site is detected in CHAPS-solubilized fractions. Similar results have recently been reported using rat brain tissues (Morishima et al., 1989). This suggests that CHAPS/NaCl solubilization of multiple states of membrane-bound [^{3}H]SP receptors shifted these sites to a single, higher-affinity state. This is supported further by the finding that [^{3}H]SP binding in CHAPS-solubilized fractions was at least 100-fold more sensitive to competition by GTP and Gpp(NH)p than membrane-bound [^{3}H]SP sites. Consequently, it appears that under the assay conditions used here, it was possible to solubilize the NK-1 receptor complex coupled to its GTP binding protein, the putative site of action of guanine nucleotides. It would now be of interest to determine if other NK-1 regulatory mechanisms [e.g., cations such as Mg^{2+} (Lee et al., 1983; Morishima et al., 1989)] are still effective in CHAPS-solubilized porcine striatal tissues.

In summary, NK-1/SP receptors present in porcine striatal tissues can be solubilized with a high yield using a mixture of the detergent CHAPS and NaCl. Moreover, the NK-1 receptor is enriched with various carbohydrate moieties including *N*-acetylglucosamine–galactose, β-galactose, mannose, and *N*-acetylglucosamine residues. Since the presence of complex *N*-acetylglucosamine–galactose moieties and β-galactose residues have not been reported for various other members of the rhodopsin receptor superfamily, it will be of interest to determine if this is relevant to specific NK-1 receptor functions.

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Modulatory Role of Glutathione on μ-Opioid, Substance P/Neurokinin-1, and Kainic Acid Receptor Binding Sites

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Abstract: Reduced glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) is an endogenous tripeptide involved in the formation and maintenance of protein thiol groups as well as in various detoxification reactions. Because multiple receptor types contain thiol groups or disulfide bridges, effects of GSH treatments on μ-opioid, neurokinin-1/substance P, and kainic acid receptor binding sites were investigated and compared with those produced by dithiothreitol (DTT), a potent synthetic reducing agent. GSH inhibited binding more potently than did DTT at all three receptor types in porcine striatal membrane homogenates as well as in CHAPS-solubilized preparations of the μ and neurokinin-1 sites. GSH-induced inhibitory effects were associated with decreases in maximal binding capacity (B_{max}) without significant alteration in apparent affinity (K_D). Cysteine, the functional moiety of GSH, mimicked GSH effects albeit with lower potencies, whereas oxidized glutathione had no effects at similar concentrations. In CHAPS-solubilized preparations, the combination of low concentrations of GSH and guanylylimidodiphosphate markedly decreased the B_{max} values of the binding of [^{3}H][D-Ala^2,Gly-ol^5]enkephalin and [^{3}H]substance P. This GSH-mediated mechanism may be important to prevent cell overstimulation by accelerating receptor uncoupling, desensitization, and/or internalization. This is in keeping with purported roles of GSH related to the maintenance of cellular integrity. **Key Words:** Glutathione—μ-Opioid—Substance P—Kainate receptors. **Liu Y. F. and Quirion R.** Modulatory role of glutathione on μ-opioid, substance P/neurokinin-1, and kainic acid receptor binding sites. *J. Neurochem.* **59**, 1024–1032 (1992).

Reduced glutathione (γ-L-glutamyl-L-cysteinylglycine; GSH) is a ubiquitous tripeptide present in peripheral and brain tissues (Reichelt and Fonnum, 1969; Meister and Anderson, 1983; Vali Pasha and Vijayan, 1989; Kudo et al., 1990). It is synthesized by the consecutive action of γ-glutamylcysteine and GSH synthetases (Meister and Anderson, 1983). Functionally, GSH is involved in reactions associated with the protection against oxidative damage and with various detoxification mechanisms. For example, GSH controls the reduction of intracellular peroxides and free radicals and ensures the maintenance of reduced disulfide bonds (Orlowski and Karkowsky, 1976; Kosower and Kosower, 1978; Meister and Anderson, 1983; Mitchell and Russo, 1987; Meister, 1988). It is, thus, essential for preserving the integrity of the cell via the control of thiol/disulfide ratio (redox state; Orlowski and Karkowsky, 1976; Meister and Anderson, 1983) by glutathione reductase. It is known that this ratio is markedly altered in favor of the oxidized form of glutathione (GSSG) in neurological disorders such as Parkinson's disease, which appear likely to involve alterations of the oxidative process (Perry et al., 1982; Spina and Cohen, 1989).

In the CNS, recent studies (Ogita and Yoneda, 1987, 1988; Ogita et al., 1988) have suggested the existence of genuine [^{3}H]GSH binding sites, distinct from that of [^{3}H]glutamate, although GSH can also compete for [^{3}H]glutamic acid binding sites (Ogita et al., 1986). The presence of these sites on the synaptic membranes may thus suggest additional roles for GSH in the modulation of neurotransmission. Moreover, recent data suggest that GSH is secreted in the extracellular space in a Ca^{2+}-dependent manner (Zangerle et al., 1991). Because disulfide bonds and thiol groups are present in most membrane bound recep-

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Abbreviations used: CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; DAGO, [D-Ala^2,Gly-ol^5]enkephalin; DTT, dithiothreitol; Gpp(NH)p, guanylylimidodiphosphate; GSH, reduced glutathione; GSSG, oxidized glutathione; NK-1, neurokinin-1; PEG, polyethylene glycol 8000; PMSF, phenylmethylsulfonyl fluoride; SP, substance P.

tors (Gilbert, 1982) and may be essential to ensure proper binding affinity, we investigated the possible role of GSH in the modulation of binding parameters of μ-opioid, substance P (SP)/neurokinin-1 (NK-1) and kainate receptors. These three receptor classes were chosen because of their possible coupling to different second messenger systems, and the presence of disulfide bridges and/or sulfhydryl groups on their respective receptors (Simon and Groth, 1975; Hazum et al., 1979; Cox et al., 1980; Marzullo and Hine, 1980; Gregor et al., 1989; Hollmann et al., 1989; Wada et al., 1989; Yokota et al., 1989; Gioannini et al., 1990; Hershey and Krause, 1990). μ-Opioid receptors are most likely coupled to a GTP-binding protein associated with the inhibition of adenylate cyclase (Ueda et al., 1988), whereas the activation of SP/NK-1 receptor stimulates phosphoinositide turnover (Quirion and Dam, 1988), and the kainate receptor is a member of the ligand-gated ion channel receptor superfamily (Monaghan et al., 1989). Dithiothreitol (DTT), a synthetic reducing agent (Konigsberg, 1972) with twice as many sulfhydryl groups as GSH, was used for comparison. Our data reveal that GSH, and to a lesser extent DTT, decreased maximal binding capacities of all three classes of sites. Combination of GSH and the stable nucleotide guanylylimidodiphosphate [Gpp(NH)p] markedly diminish both K_D and B_{max} values of μ-opioid and SP/NK-1 sites in 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized membrane preparations. Thus, GSH may be actively involved in the modulation of receptor-mediated responses in the CNS.

MATERIALS AND METHODS

Materials

[^{3}H]SP (36.3 Ci/mmol), [^{3}H]kainic acid (60 Ci/mmol), and [^{3}H][D-Ala^2,Gly-ol^5]enkephalin ([^{3}H]DAGO) (30 Ci/mmol) were purchased from New England Nuclear Corporation (Boston, MA, U.S.A.). Gpp(NH)p was obtained from Boehringer Ingelheim (Montreal, Quebec, Canada). CHAPS was purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.) and polyethylene glycol 8000 (PEG) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Unlabeled SP was purchased from IAF Biochem Inc. (Laval, Quebec, Canada). GSSG, GSH, DTT, cysteine, protease inhibitors, and all other chemicals of general use were purchased from Sigma Chemical Co. Whatman GF/B filters and Aquassure scintillation cocktail were obtained from Fisher Canada Ltd. (Montreal, Quebec, Canada). Porcine brains were obtained fresh from a local slaughterhouse (R. Boucher, St-Jean Baptiste de Rouville, Quebec, Canada).

Membrane preparations

Fresh pig brains were dissected on ice and striatal membranes were prepared as follows: Porcine striata were homogenized (Brinkmann Polytron at setting 6) in 50 m*M* ice-cold Tris-HCl (pH 7.4) buffer containing 150 m*M* NaCl, 150 m*M* KCl, 12 m*M* EDTA, 200 μ*M* phenylmethylsulfonyl fluoride (PMSF), 40 μg/ml of bacitracin, 4 μg/ml of leupeptin, and 2 μg/ml of chymostatin. These homogenates were then incubated for 30 min at 4°C before being centrifuged at 30,000 *g* for 20 min at 4°C and washed twice (five times for [^{3}H]kainic acid assay) with 50 m*M* Tris-HCl (pH 7.4) buffer. Pellets were resuspended in 0.32 *M* sucrose containing 200 μ*M* PMSF and 40 μg/ml of bacitracin before storage at −80°C until use.

Binding assays in membrane homogenates

For all three binding assays, 60-min incubations were performed at room temperature in 50 m*M* Tris-HCl (pH 7.4) buffer containing various concentrations of either [^{3}H]SP, [^{3}H]DAGO, or [^{3}H]kainic acid, and 5 m*M* $MgSO_4$, 40 μg/ml of bacitracin, 4 μg/ml of leupeptin, and 2 μg/ml of chymostatin in the presence of 0.5–1.0 mg of membrane protein (Bradford, 1976) in a final volume of 1 ml. Total and nonspecific binding were determined in triplicate in the absence or presence of either 1.0 μ*M* unlabeled SP, 1.0 μ*M* naloxone, or 10.0 μ*M* kainic acid. Incubations were terminated by adding 4 ml of ice-cold 50 m*M* Tris-HCl (pH 7.4) buffer and membranes were then filtered (Cell Harvester, Brandel Instruments, Gaithersburg, MD, U.S.A.) on Whatman GF/B glass-fiber filters that had been presoaked in 0.5% polyethyleneimine for a minimum of 3 h before use to reduce absorption. Filters were then washed three times (5 ml each) using ice-cold Tris-HCl (pH 7.4) buffer, and bound radioactivities were determined using a Beckman liquid scintillation counter at an efficiency of 45%. Under these assay conditions, specific [^{3}H]SP and [^{3}H]DAGO binding represented between 70 and 80% of total binding at concentrations close to K_D values, whereas ≤90% of ligand was specifically bound in [^{3}H]kainic acid assays. Saturation and competition data were analyzed using a computerized nonlinear least squares curve-fitting program (Bio-Soft Elsevier, U.K.).

Solubilization of [^{3}H]DAGO and [^{3}H]SP binding sites

Solubilization was performed as described in detail elsewhere (Liu and Quirion, 1991). Membrane preparations were centrifuged and pellets resuspended in 50 m*M* Tris-HCl (pH 7.4) buffer containing 10 m*M* CHAPS, 1 *M* NaCl, and 100 μ*M* PMSF to yield a protein concentration of 3 mg/ml. This mixture was then shaken in an ice bath for 1 h before centrifugation at 100,000 *g* for 1 h. Pellets were discarded and supernatants were used as the source of solubilized [^{3}H]DAGO and [^{3}H]SP receptor proteins.

Binding assay in CHAPS-solubilized fractions

Solubilized membrane preparations were mixed 1:1 in 40% PEG and centrifuged at 30,000 *g* for 20 min (Liu and Quirion, 1991). Resulting pellets were resuspended in 50 m*M* Tris-HCl (pH 7.4) buffer. Receptor binding assays were then performed for 60 min at room temperature in a 50 m*M* Tris-HCl (pH 7.4) buffer containing various concentrations of either [^{3}H]DAGO or [^{3}H]SP and 5 m*M* $MgSO_4$, 2 μg/ml of chymostatin, 40 μg/ml of bacitracin, and 4 μg/ml of leupeptin to give a final volume of 1 ml. Total and nonspecific binding was determined as for membrane homogenates. Incubations were terminated by the addition of 4 ml of ice-cold 15% PEG buffer containing 0.1 ml of 0.02% γ-globulin solution, vortexed and filtered on Whatman GF/B glass-fiber filters presoaked in 0.5% polyethyleneimine. Filters were then washed once using 5 ml of PEG (7.5%). Radioactivity was evaluated as described for membrane binding. Under such assay conditions, specific [^{3}H]SP and [^{3}H]-DAGO binding comprised ~80% of total binding at concentrations close to K_D values. [^{3}H]Kainic acid binding pro-

tein was not adequately solubilized using this protocol (not shown).

Treatments with reducing agents

Unless indicated otherwise, membrane homogenates or CHAPS-solubilized preparations were preincubated in 50 m*M* fresh Tris-HCl buffer containing 5 m*M* $MgSO_4$ and peptidase inhibitors (see above) for 10 min at 25°C in the presence of various concentrations of either GSH, DTT, cysteine, or GSSG. At the end of this period, homogenates or CHAPS-solubilized preparations were incubated as described above using freshly prepared buffer and reagents.

Reversibility experiments

In another series of experiments, the reversibility of GSH effects was tested by extensively washing (eight to 10 washes, with correction of values for protein losses) preincubated membranes to remove reducing agent. After the preincubation with 10 m*M* GSH, membranes were centrifuged and pellets extensively washed with 50 m*M* Tris-HCl (pH 7.4) buffer at 25°C without the reducing agent, followed by incubations with radioligands as described above.

Protein assays

Protein concentrations were determined using a modified Coomassie Blue assay with γ-globulin as standard (Bradford, 1976).

Statistical analysis

Statistical significance was determined using Student's *t* test, and $p < 0.05$ considered statistically significant.

RESULTS

Effects of GSH and DTT on [^{3}H]DAGO binding in membrane and CHAPS-solubilized preparations

It has been reported that DTT affects the migration of a μ-opioid–related binding protein in sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (Gioannini et al., 1990). Accordingly, we observed that DTT inhibited [^{3}H]DAGO binding in this study (Fig. 1). However, when GSH was used, a more potent inhibitory action was observed, in accordance with an earlier study (Cox et al., 1980). As shown in Fig. 1A, specific [^{3}H]DAGO binding was totally abolished by 10 m*M* GSH (IC_{50}, 4.3 ± 1 m*M*; Table 1). DTT was much weaker with an IC_{50} of 65 ± 11 m*M*, which is ~15-fold less potent than GSH (Fig. 1A; Table 1). Cysteine has intermediate potency with an IC_{50} of 14 ± 3 m*M* (Table 1), whereas GSSG was inactive at ≤100 m*M*. The removal of GSH by extensive washes before incubation reversed the inhibitory effects of the preincubation with GSH (10 m*M*) on [^{3}H]DAGO binding (91 ± 6% of control values, n = 4).

To test for the nature of the inhibitory action of these agents on specific [^{3}H]DAGO/μ-opioid binding, saturation experiments were performed with or without pretreatment of membrane homogenates with various concentrations of GSH or DTT. As shown in Fig. 2A and B, a single class of high-affinity [^{3}H]-DAGO binding sites was detected in porcine striatal membrane preparations with an apparent affinity (K_D) of 1.2 ± 0.2 n*M* and a maximal capacity (B_{max}) of 53 ± 8 fmol/mg of protein (n = 4). Pretreatment of membranes with 5 m*M* GSH reduced B_{max} by 60% ($p < 0.005$) without significantly altering K_D values (Fig. 2A, B). Similarly, 100 m*M* DTT decreased B_{max} by 30% ($p < 0.05$) without modifying K_D (Fig. 2A, B).

To characterize further the effects of GSH and DTT on specific [^{3}H]DAGO/μ-opioid binding, we evaluated their efficacies in CHAPS-solubilized porcine striatal preparations. As shown in Table 1, GSH, cysteine, and DTT acted on specific [^{3}H]DAGO bind-

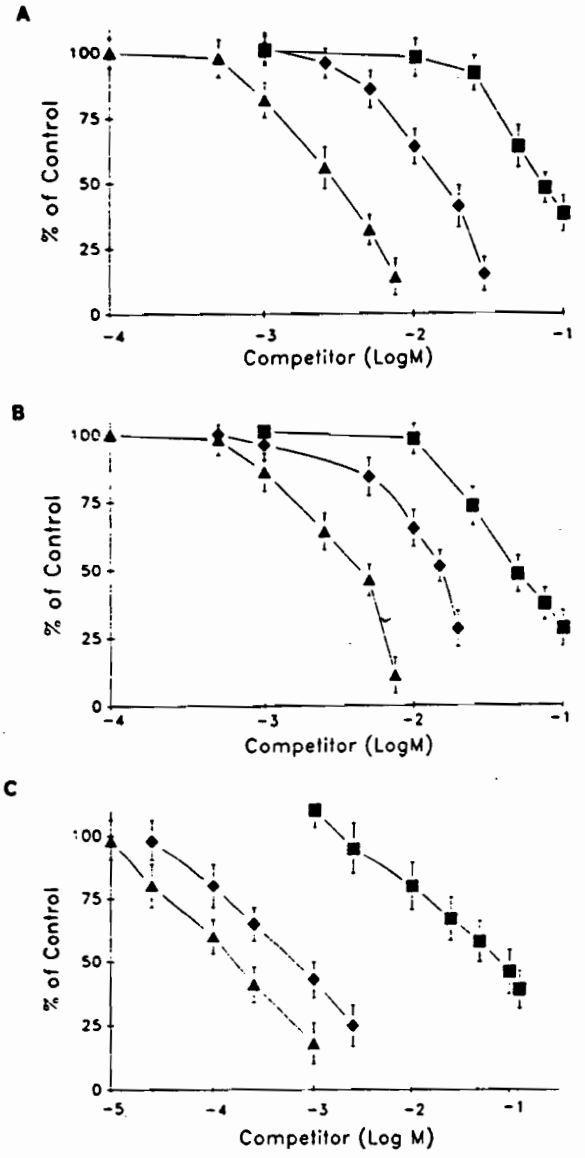

FIG. 1. Concentration-dependent inhibitory effects of GSH (▲), cysteine (♦), and DTT (■) on specific [^{3}H]DAGO (A), [^{3}H]SP (B), and [^{3}H]kainic acid (C) binding in porcine striatal membrane preparations. Competitors were added 10 min before incubation. Concentrations of 2.5 n*M* [^{3}H]DAGO, 1.1 n*M* [^{3}H]SP, and 3.0 n*M* [^{3}H]kainic acid were used in the respective assay. Data are the mean ± SEM of at least four experiments (each performed in triplicate) for each binding assay.

TABLE 1. *Comparative potencies of GSH, cysteine, DTT, and GSSG on [³H]DAGO/μ, [³H]SP/neurokinin-1, and [³H]kainic acid binding in porcine striatal membranes or CHAPS-solubilized preparations*

	IC_{50} (m*M*)				
	[³H]DAGO		[³H]SP		[³H]Kainic acid
Reducing agent	Membrane	Soluble	Membrane	Soluble	membrane
GSH	4.3 ± 1.0	2.1 ± 1.0	5.1 ± 1.2	2.3 ± 0.8	0.22 ± 0.1
Cysteine	14 ± 3.0	10 ± 3.0	17 ± 4.0	15 ± 3.0	1.1 ± 0.5
DTT	65 ± 11	36 ± 9.0	42 ± 13	102 ± 18	51 ± 8.0
GSGG	—	—	—	—	0.5 ± 0.1

Values are the mean ± SEM of three separate experiments, each performed in triplicate. IC_{50} values represent the concentrations of reducing agents needed to compete for 50% of specifically bound radioligands. GSSG was inactive on [³H]DAGO and [³H]SP binding at ≤100 m*M*.

ing to CHAPS solubilized, PEG-precipitated membrane preparations. For all three agents, relative potencies were greater in soluble preparations with IC_{50} values ranging between 2.1 ± 1 and 36 ± 9 m*M*. As for membrane homogenates, pretreatments with GSH or DTT markedly decreased B_{max} values (without affecting K_D) in CHAPS-solubilized preparations (Fig. 2C and D). Whereas values of 1.4 ± 0.3 n*M* and 31 ± 4 fmol/mg of protein were determined in control preparations, K_D of 1.2 ± 0.2 n*M* and 1.4 ± 0.5 n*M* and B_{max} of 18 ± 3 fmol/mg of protein ($p < 0.005$) and 23 ± 2 fmol/mg of protein were derived after GSH and DTT pretreatment, respectively.

Effects of GSH and DTT on [³H]SP binding in membrane and CHAPS-solubilized preparations

To investigate whether the role of GSH on μ-opioid receptor binding parameters is unique, we tested next its effects on [³H]SP/NK-1 binding sites, which are also known to be highly enriched in striatal tissues

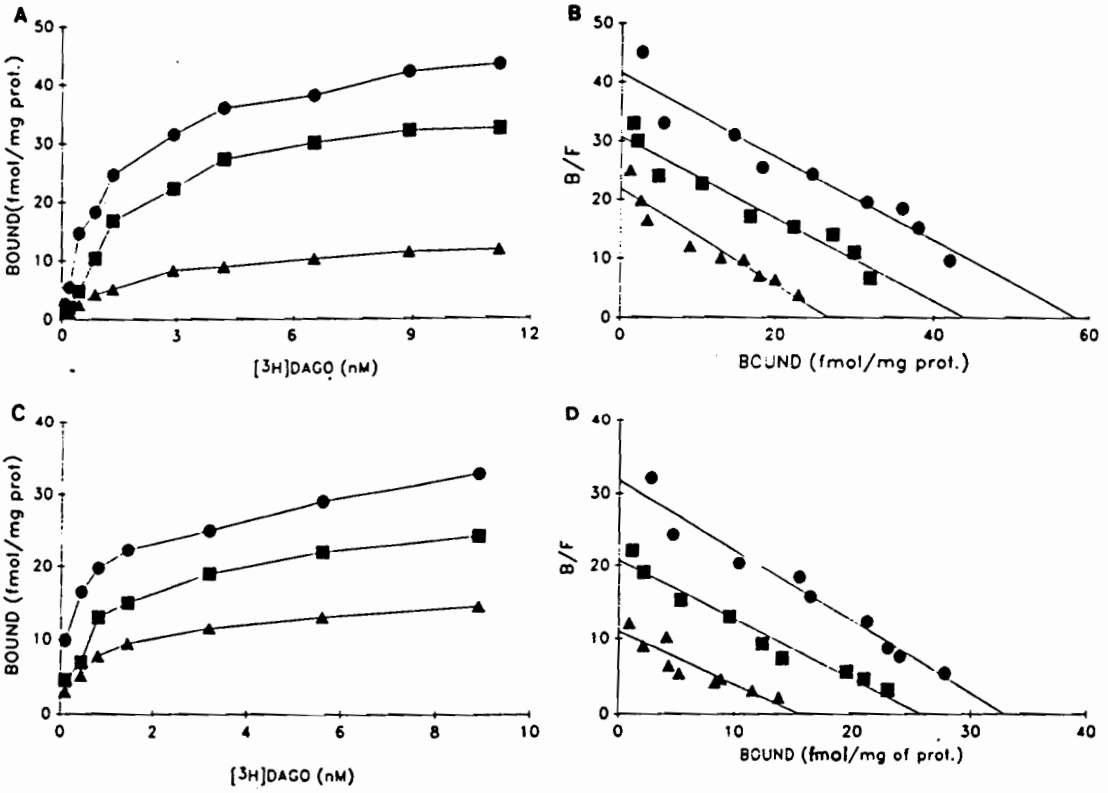

FIG. 2. Saturation curves (**A, C**) and Scatchard representations (**B, D**) of specific [³H]DAGO/μ-opioid binding to porcine striatal membrane homogenates (A, B) and CHAPS-solubilized fractions (C, D). Membrane homogenates and CHAPS-solubilized preparations were preincubated for 10 min at room temperature in the absence (control, ●) or in the presence of either 2.0 (soluble) or 5.0 (homogenate) m*M* GSH (▲) or 100 m*M* DTT (■) followed by incubations in the presence of increasing concentrations (0.05–11 n*M*) of [³H]DAGO as described in Materials and Methods. Values represent the mean of three to four separate experiments in each case.

(Quirion and Dam, 1988). As for [^{3}H]DAGO binding, GSH, cysteine, and DTT inhibited specific [^{3}H]SP binding to both membrane (Fig. 1B) and CHAPS-solubilized (Table 1) receptor preparations. The order of potency was GSH > cysteine > DTT in both preparations as observed for [^{3}H]DAGO (Table 1). GSSG was inactive at ≤100 m*M*. The potencies of GSH and cysteine were slightly greater in soluble preparations, whereas DTT was more potent in membrane homogenates than in soluble preparations (Table 1). Extensive washes of membranes after preincubation reversed the effects of 10 m*M* GSH on [^{3}H]SP binding (86 ± 11% of control values; n = 3).

Pretreatments with GSH and DTT were associated with decreases in B_{max} values in both membrane homogenates (not shown) and CHAPS-solubilized preparations (Fig. 3). GSH (2 m*M*) induced a 50% loss in number of sites (from 78 ± 6 to 36 ± 4 fmol/mg of protein; $p < 0.001$) without alteration of K_D values (1.0 ± 0.4 to 1.2 ± 0.2 n*M*) in CHAPS-solubilized preparations (Fig. 3). Pretreatment with 100 m*M* DTT decreased B_{max} by ~25% (57 ± 5 fmol/mg of protein, $p < 0.05$) without changing K_D (1.4 ± 0.3 n*M*) in this same preparation (Fig. 3).

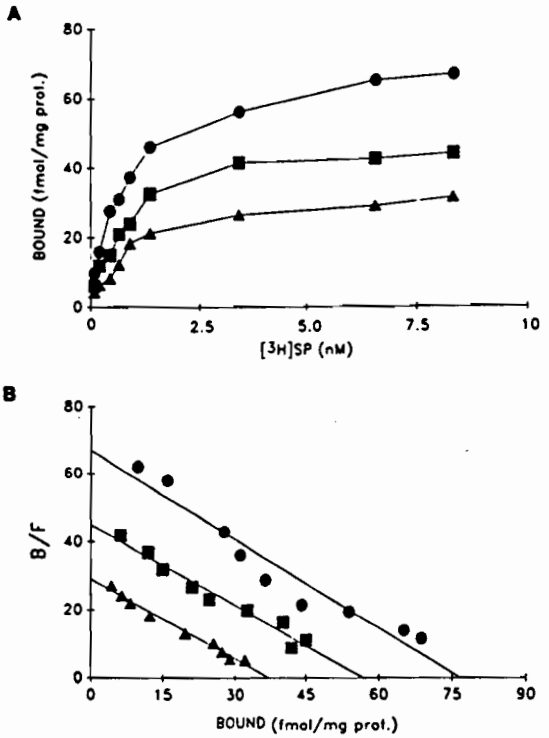

FIG. 3. Saturation curve (A) and Scatchard representation (B) of specific [^{3}H]SP/neurokinin-1 binding to CHAPS-solubilized porcine striatal preparations. CHAPS-solubilized membrane preparations were preincubated for 10 min at room temperature in the absence (control, ●) or in the presence of either 2.0 m*M* GSH (▲) or 100 m*M* DTT (■) followed by incubations in the presence of increasing concentrations (0.05–11.0 n*M*) of [^{3}H]SP as described in Materials and Methods. Values represent the mean of three separate experiments in each case.

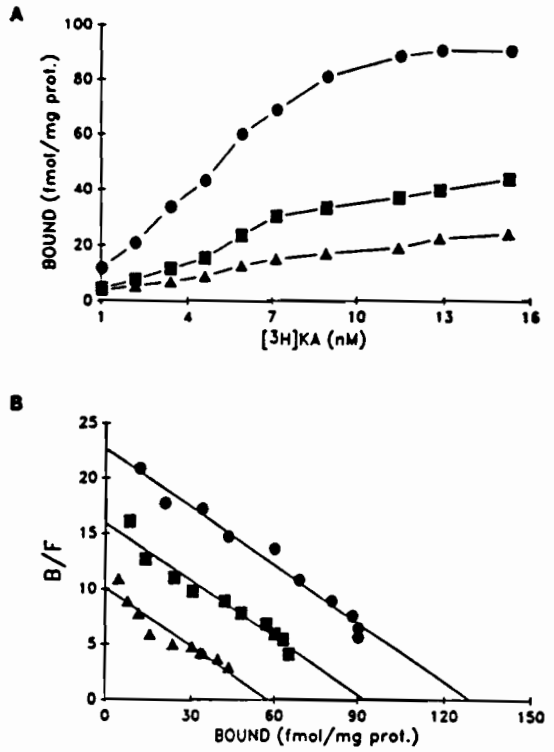

FIG. 4. Saturation curve (A) and Scatchard representation (B) of specific [^{3}H]kainic acid ([^{3}H]KA) binding to porcine striatal membrane homogenates that were preincubated for 10 min at room temperature in the absence (control; ●) or in the presence of either 0.5 m*M* GSH (▲) or 50 m*M* DTT (■) followed by incubations in the presence of increasing concentrations (1–20 n*M*) of [^{3}H]-kainic acid as described in Materials and Methods. Values represent the mean of three separate experiments in each case.

Effects of GSH and DTT on [^{3}H]kainic acid binding in membrane homogenates

Because the functional moiety of GSH is the amino acid, cysteine, and because it also contains glutamate, it may be possible that GSH has greater access to binding sites for amino acid than for neuropeptide receptors. To test this hypothesis, we investigated effects of GSH, cysteine, and DTT on [^{3}H]kainic acid binding sites in porcine striatal membrane homogenates. As shown in Fig. 4, [^{3}H]kainic acid apparently binds to a single class of high-affinity (K_D, 5.5 ± 0.8 n*M*), low-capacity (B_{max} = 123 ± 23 fmol/mg of protein) sites in this tissue. As seen for [^{3}H]DAGO and [^{3}H]SP binding, GSH, cysteine, and DTT dose-dependently inhibited [^{3}H]kainic acid binding in porcine striatal homogenates (Table 1; Fig. 1C). Both GSH and cysteine were significantly more potent (>10-fold) against [^{3}H]-kainic acid binding sites than on the two neuropeptide receptor classes studied here (Table 1). The relative potency of DTT was similar in all assays (Table 1), revealing differences in the capacity of endogenous and exogenous reducing agents to modulate receptor binding parameters. Moreover, GSSG exhibited high potency on [^{3}H]kainic acid binding sites with an IC_{50}

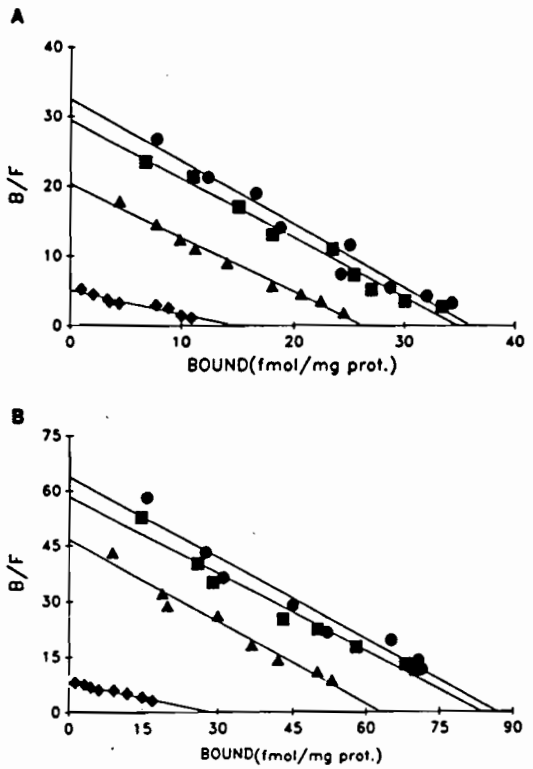

FIG. 5. Scatchard representations of [^{3}H]DAGO/μ-opioid (A) and [^{3}H]SP/neurokinin-1 (B) binding to CHAPS-solubilized porcine striatal membrane fractions in the absence (●) or the presence of either 1.0 μM Gpp(NH)p (■) or 1.0 mM GSH (▲), or a mixture of both at these concentrations (◆). CHAPS-solubilized fractions were preincubated for 10 min at room temperature in the presence of 1.0 mM GSH when appropriate, followed by incubations with either [^{3}H]DAGO (0.1–10 nM) or [^{3}H]SP (0.05–8.0 nM) as described in Materials and Methods. Values represent the mean of at least three experiments in each case.

of 0.5 ± 0.1 mM (n = 4), whereas, as noted above, it was not a potent inhibitor of peptide bindings.

As shown in Fig. 4, the effects of both GSH and DTT on [^{3}H]kainic acid binding were related to decreases in maximal binding capacities. Pretreatment of membrane homogenates with 0.5 mM GSH decreased B_{max} values by 46% to 68 ± 11 fmol/mg of protein ($p < 0.005$), whereas 50 mM DTT reduced B_{max} to 95 ± 14 fmol/mg of protein. K_D values were not significantly altered by either treatment (GSH, 6.5 ± 1.0 nM; DTT, 5.8 ± 0.7 nM). Effects of reducing agents on soluble [^{3}H]kainic acid binding protein were not investigated because of poor receptor recovery in CHAPS-solubilized preparations.

Interactions between GSH and Gpp(NH)p on [^{3}H]DAGO and [^{3}H]SP binding sites in CHAPS-solubilized membrane preparations

Both μ-opioid and SP/NK-1 receptors are G protein–coupled receptors (Ueda et al., 1988; Yokota et al., 1989; Hershey and Krause, 1990), and various studies have shown that GTP or its nonhydrolyzable analogue, Gpp(NH)p, inhibited binding of various radioligands to these two receptors by decreasing their affinities (Chang et al., 1981; Lee et al., 1983; Gioannini et al., 1985; Tanaka et al., 1986; Childers, 1988). Similar results were obtained here in CHAPS-solubilized porcine striatal preparations at concentrations greater than 1.0 μM Gpp(NH)p (data not shown). To investigate possible interactions between GSH and Gpp(NH)p, we evaluated the effects of 1 μM Gpp(NH)p on [^{3}H]DAGO and [^{3}H]SP binding, in the absence or presence of a low concentration of GSH (1.0 mM). As shown in Fig. 5, 1.0 μM Gpp(NH)p did not significantly alter specific [^{3}H]DAGO (Fig. 5A) or [^{3}H]SP (Fig. 5B) binding, whereas pretreatment with GSH, by itself, decreased B_{max} values by ~20–30% (Table 2). Combination of both Gpp(NH)p and GSH markedly altered both K_D and B_{max} values for [^{3}H]-DAGO as well as [^{3}H]SP binding to CHAPS-solubilized membrane preparations (Fig. 5; Table 2). Interactions can thus be seen between GSH and nucleotides in the modulation of receptor binding parameters.

DISCUSSION

In this study, μ-opioid, SP/NK-1, and kainic acid receptor binding sites were found to be reversibly affected by physiological concentrations of GSH. At these concentrations, GSH markedly decreased maxi-

TABLE 2. *Binding characteristics of [^{3}H]DAGO and [^{3}H]SP sites in CHAPS-solubilized porcine striatal fractions after treatments with GSH or Gpp(NH)p*

Treatment	[^{3}H]DAGO		[^{3}H]SP	
	K_D (nM)	B_{max} (fmol/mg of protein)	K_D (nM)	B_{max} (fmol/mg of protein)
Control	1.4 ± 0.3	36 ± 8	1.0 ± 0.3	82 ± 11
Gpp(NH)p (1 μM)	1.6 ± 0.6	34 ± 9	1.4 ± 0.7	84 ± 8
GSH (1 mM)	1.5 ± 0.7	28 ± 7	1.3 ± 0.9	58 ± 11[a]
GSH (1 mM) plus Gpp(NH)p (1 μM)	4.1 ± 1.2[a]	16 ± 7[b]	3.1 ± 1.1[a]	28 ± 9[b]

Values are the mean ± SD of three separate experiments, each performed in triplicate.
[a] $p < 0.05$; [b] $p < 0.005$, compared with control values.

mal binding capacities of these three classes of sites in porcine striatal membrane homogenates and of the peptide binding to CHAPS-solubilized preparations. These effects were mimicked by cysteine and a synthetic reducing agent, DTT, although at higher concentrations. Thus, these three classes of receptors contain reactive disulfide bridges and/or thiol groups, in accordance with earlier reports (see introductory section). The integrity of these moieties appears essential for maximal binding capacities probably by ensuring adequate three-dimensional receptor conformation (Meister and Anderson, 1983). Our data also reveal that treatments with GSH and other reducing agents did not significantly alter receptor binding affinities. Therefore, it is likely that, after treatments with a reducing agent, a certain proportion of sites are reversibly denatured rendering them unable to recognize the relevant ligand (hence decreased B_{max} values), whereas the other portion is not significantly affected and can still bind the radioligand without evident change in K_D. The data also suggest that thiol groups or disulfide bridges are probably closely associated with binding sites of the receptor complex. Moreover, the reversible nature of GSH effects on the three types of receptors studied indicates that B_{max} losses are apparent and not associated with permanent alterations of the globular structure of the receptors.

The recent cloning of SP/NK-1 (Yokota et al., 1989; Hershey and Krause, 1990) and kainate (Gregor et al., 1989; Hollmann et al., 1989; Wada et al., 1989) receptors should lead to further understanding of the structural and functional features of these receptors, especially in regard to the role of cysteine residues and disulfide bridges. Both transmembrane and intracellular domains of NK-1 and kainate receptors contain highly conserved regions enriched in cysteine residues (Gregor et al., 1989; Hollmann et al., 1989; Wada et al., 1989; Yokota et al., 1989; Hershey and Krause, 1990). It has been suggested that cysteine moieties may be involved in the maintenance of adequate binding parameters and/or coupling to second messenger systems by stabilization of certain structural features of the receptor domain (Gilbert, 1982). This has been most extensively studied for other receptor types such as the nicotinic cholinergic (Moore and Raftery, 1979; Walker et al., 1981) and the β-adrenergic (Lucas et al., 1978; Vauquelin et al., 1979) receptors. Changes in receptor binding affinities were reported in most of these earlier studies (Lucas et al., 1978; Moore and Raftery, 1979; Walker et al., 1981). However, as in the present report, Vauquelin et al. (1979) observed decrements in binding capacities after DTT and GSH treatments. It would be of interest to determine whether mechanisms involved in alterations of binding affinities are identical to those implicated in losses of maximal binding capacities or are related to different actions of reducing agents.

The fact that μ-opioid, NK-1, and kainic acid receptor binding sites are all affected (mostly B_{max}, not K_D) by reducing agents suggests that the effectiveness of these agents is not dependent on receptor coupling mechanisms and second messenger systems. Whereas NK-1 (Yokota et al., 1989; Hershey and Krause, 1990) and, most likely, μ-opioid (Barnard and Demoliou-Mason, 1983; Ueda et al., 1988) receptors are members of the G protein–coupled receptor superfamily, kainate receptors are members of the ligand-gated ion channel group (Gregor et al., 1989; Hollmann et al., 1989; Monaghan et al., 1989; Wada et al., 1989). Thus, the effects of GSH and cysteine are probably broadly applicable to different receptor families. However, we observed some difference in the potencies of various reducing agents. Although the order of potency of GSH, cysteine, and DTT was similar (GSH > cysteine > DTT) in all binding assays, GSH and cysteine, but not DTT, were significantly more active on [^{3}H]kainic acid binding than in the two other assays.

The oxidized form of glutathione, GSSG, was inactive at investigated concentrations on [^{3}H]DAGO and [^{3}H]SP binding sites. This suggests that the sulfhydryl groups of GSH and cysteine are mediating the interactions with these two receptor classes. However, GSSG was quite active on [^{3}H]kainic acid binding sites. It may be that the mechanism of action of GSH and congeners (GSSG and cysteine) is different on this receptor class, thus explaining the different effects of DTT on [^{3}H]kainic acid sites. However, it is unlikely to involve direct competition, as reported earlier for L-[^{3}H]glutamate binding sites (Ogita et al., 1988) because no change in affinity was observed in competition assays. Further experiments will be necessary to clarify this issue.

The most intriguing finding of the present study relates to the combined effects of GSH and Gpp(NH)p on [^{3}H]DAGO and [^{3}H]SP binding in CHAPS-solubilized membrane preparations. Although it is well established that GTP and its metabolically stable derivative Gpp(NH)p can inhibit, by decreasing receptor affinity, μ-opioid (Chang et al., 1981; Gioannini et al., 1985; Childers, 1988) and NK-1 (Lee et al., 1983; Tanaka et al., 1986) binding in membrane homogenates and solubilized preparations, combination of a reducing agent such as GSH and nucleotides was not evaluated in these earlier studies. We observed that Gpp(NH)p potentiated the action of GSH in reducing the B_{max} value of the binding of [^{3}H]DAGO and [^{3}H]SP. The exact mechanism(s) involved here remains to be established. However, a possible explanation is that GSH alters receptor conformation (possibly by a direct action on G proteins, at least in the case of μ and NK-1 receptors) such that it promotes the formation of uncoupled, lower-affinity receptor states, which in turn induces greater decrements in apparent maximal binding capacities. If similar potentiation between endogenous reducing agents (GSH and cysteine) and nucleotides are operant in vivo, it could have important functional implication

in signal transduction associated with receptor activation. This could be especially relevant to ensure the termination of receptor activity. This would also be in keeping with better known roles of GSH that relate to detoxification processes and protection of cellular integrity (Orlowski and Karkowsky, 1976; Kosower and Kosower, 1978; Meister and Anderson, 1983; Mitchell and Russo, 1987).

The interaction of GSH with receptor-mediated effects could have significance in various pathological conditions involving neurodegeneration. For example, in patients with Parkinson's disease, GSH levels are markedly decreased in mesencephalic areas (Perry et al., 1982; Spina and Cohen, 1989) with compensating increases in GSSG; this could lead to modification of receptor redox states, oxidative stress, cell exhaustion, and ultimately, cell death. Current interest in Parkinson's disease relates to the use of monoamine oxidase inhibitors to restore GSH levels and hence diminish oxidation, peroxide formation, and cell death (Spina and Cohen, 1989). It is possible that extrastriatal degenerative disorders could also be associated with similar dysfunctions; for example, in cortical cell cultures, it has been shown recently that reduced levels of GSH cause increased excitotoxicity (Bridges et al., 1991). The results of the present study suggesting that GSH, in conjunction with nucleotides, may be involved in the modulation of receptor responsiveness is in accordance with the general roles of endogenous reducing agents in controlling cellular stress.

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* Pg.- Error --
* Pg.- # 195 ?
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Editor
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May 25, 1993

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Dear Dr. Means:

I would like to ask your permission to reproduce the article "Cholera toxin-sensitive 3',5'-cyclic adenosine monophosphate and calcium signals of the human dopamine-D1 receptor: selective potentiation by protein kinase A" published on Mol. Endo. 6: 1815-1824 for my PhD thesis preparation. I am looking forward your reply. I would like to thank you in advance.

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Ya Fang Liu MD

OK as requested
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June 6, 1993

Ya Fang Liu, M.D.
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Dear Dr. Liu:

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3. Y.F. Liu & R. Quirion (1992) Modulatory role of glutathione on u opioid, substance P/neurokinin 1, and kainic acid receptor binding sites. 59:1024-1032.

Please acknowledge appropriately the Journal of Neurochemistry. Good luck in your writing.

Yours sincerely,

Alan A. Boulton, Ph.D., D.Sc., F.R.S.C.
Director, Neuropsychiatric Research Unit
and Professor, Department of Psychiatry

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